Evaluation Report

proficiency test

DLA ptAL03 (2021)

Allergens III:

β-Lactoglobulin, Casein und Gluten

in hypoallergenic Infant Food with hydrolysed Milk Protein

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1st Correction 27/09/2021:
In the overview table of the z-scores (p. 59), the z-scores of participants 7 and 9 were not transferred or incorrectly transferred from the results section for beta-lactoglobulin in the spiking level sample. This has been corrected.

Allgemeine Informationen zur Eignungsprüfung (EP)
General Information on the proficiency test (PT)

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EP-Koordinator *PT-Coordinator*	Dr. Matthias Besler-Scharf
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EP-Bericht Freigabe *PT-Report Authorization*	Dr. Matthias Besler-Scharf (Technischer Leiter / Technical Manager) *- gezeichnet / signed M. Besler-Scharf* Alexandra Scharf MSc. (QM-Beauftragte / Quality Manager) *- gezeichnet / signed A. Scharf* Datum / Date: 24 September 2021
Unteraufträge *Subcontractors*	Im Rahmen dieser Eignungsprüfung wurden nachstehende Leistungen im Unterauftrag vergeben: Homogenitätsprüfung der EP-Parameter, Proteinbestimmung As part of the present proficency test the following services were subcontracted: Homogeneity tests of PT-parameter(s), protein determination
Vertraulichkeit *Confidentiality*	Die Teilnehmerergebnisse sind im EP-Bericht in anonymisierter Form mit Auswertenummern benannt. Daten einzelner Teilnehmer werden ausschließlich nach vorheriger Zustimmung des Teilnehmers an Dritte weitergegeben. Participant result are named anonymously with evaluation numbers in the PT report. Data of individual participants will be passed on to third parties only with prior consent of the participant.
Akkreditierung *Accreditation* ilac-MRA DAkkS Deutsche Akkreditierungsstelle D-EP-21534-01-00	nach / according to: ISO/IEC 17.043-2010 Konformitätsbewertung - Allgemeine Anforderungen an Eignungsprüfungen Conformity Assessment - General Requirements for Proficiency Testing Die Akkreditierung gilt für den in der Urkundenanlage genannten Umfang. The accreditation is valid for the scope of the annex to the certificate of accreditation

Contents

1. Introduction

The participation in proficiency testing schemes is an essential element of the quality-management-system of every laboratory testing food and feed, cosmetics and food contact materials. The implementation of proficiency tests enables the participating laboratories to prove their own analytical competence under realistic conditions. At the same time they receive valuable data regarding the verification and/or validation of the particular testing method [1, 5].
The purpose of DLA is to offer proficiency tests for selected parameters in concentrations with practical relevance.
Realisation and evaluation of the present proficiency test follows the technical requirements of DIN EN ISO/IEC 17043 (2010) and DIN ISO 13528:2009 / ISO 13528:2015 [2, 3].

2. Realisation

2.1 Test material

Two PT-samples with the same food matrix were provided for the detection and quantitative determination of the allergens in the range of mg/kg as well as one spiking level sample with a simple matrix. One of the samples (spiked sample) and the spiking level sample contain the respective allergenic ingredients in a similar concentration range. The results of the spiking level sample should give the possibility of a comparison with the spiked sample in respect to the detectability of the allergens with and without the influence of matrix and / or food processing.

The test material of the food matrix samples is a mixture of commercially available infant formulas with soy protein and hydrolyzed whey protein (powder for preparation) for special medical purposes (balanced dietetic foods), e.g. for cow's milk allergy with further addition of casein hydrolyzate.
The basic composition of both sample A and sample B was the same (see table 1).
After homogenization of the basic mixture the **spiked sample B** was produced as follows:
The spiking materials containing the allergenic ingredients skimmed milk powder, whey powder and wheat flour were crushed and sieved by a centrifugal mill (mesh <250 µm or <500 µm), added to an aliquot of the basic mixture and the mixture was homogenized. Subsequently, the basic mixture was again added in additional steps and homogenized in each case until the total quantity had been reached.
The **spiking level sample** was produced with the allergenic compounds above mentioned by multi-stage addition of potato powder (mesh 500 µm) and homogenization.

The samples A and B were portioned to approximately 25 g into plastic containers, the spiking levels sample to approximately to 15 g in metallized PET film bags.

Table 1: Composition of DLA-Samples

Ingredients	**Sample A**	**Sample B**	**Spiking Level Sample**
Infant formula with soy protein, in case of intolerance or allergy to milk, from birth (powder for preparation, balanced diet) Ingredients: maltodextrin, vegetable oils, soy protein isolate, vitamins, minerals and other food additives Nutrients per 100 g powder: Fat 23 g, Carbohydrates 58 g, Protein 11 g, Salt 0,5 g	57,2 g/100g	57,1 g/100g	-
Infant formula with hydrolyzed whey protein, in case of allergy to milk, from birth (powder for preparation, balanced diet) Ingredients: glucose syrup, hydrolyzed whey protein, vegetable oils, maltodextrin, fish oil, vitamins, minerals and other food additives Nutrients per 100 g powder: Fat 25 g, Carbohydrates 54 g, Protein 13 g, Salt 0,4 g	38,0 g/100g	37,9 g/100g	-
Casein-Hydrolyzate (Drink powder) Ingredientsn: Casein-Hydrolyzate Nährwertangaben pro 100 g Pulver: Nutrients per 100 g powder: Fat 1,5 g, Carbohydrates 0 g, Protein 81 g, Salt 3,8 g	4,7 g/100g	4,7 g/100g	-
thereof Milk protein hydrolyzates - hydrolyzed whey proteins - hydrolyzed caseins	 5,1 g/100g 3,8 g/100g	 5,1 g/100g 3,8 g/100g	-
Potato Powder Ingredients: Potatoes, E471, E304, E223, E100	–	–	99,8 g/100 g
Milk component 1: skimmed milk powder mixture (9 products from Europe, USA) - as skimmed milk powder* - thereof 33,0% total protein** - thereof Casein*** - thereof β-Lactoglobulin***	–	 58,8 mg/kg 19,4 mg/kg 15,5 mg/kg 1,94 mg/kg	 58,3 mg/kg 19,2 mg/kg 15,4 mg/kg 1,92 mg/kg
Milk component 2: whey powder mixture (4 products from Germany) - as whey powder * - thereof 15,9% total protein** - thereof β-Lactoglobulin***	–	 260 mg/kg 41,4 mg/kg 20,7 mg/kg	 359 mg/kg 57,0 mg/kg 28,5 mg/kg
Sum of milk components - thereof total protein - thereof Casein - *thereof β-Lactoglobulin*	–	*319 mg/kg* 60,8 mg/kg *15,5 mg/kg* *22,6 mg/kg*	*417 mg/kg* 76,2 mg/kg *15,4 mg/kg* *30,4 mg/kg*
Wheat: Wheat flour mixture (21 products from Europe, Asia, USA) - as wheat flour* - thereof 10,1% total protein** - thereof gluten***	–	 173 mg/kg 17,5 mg/kg 15,1 mg/kg	 169 mg/kg 17,1 mg/kg 14,7 mg/kg
further Ingredients:	–	<0,2 g/100 g	<0,2 g/100 g

Ingredients	Sample A	Sample B	Spiking Level Sample
Maltodextrin and silicon dioxide			

See next page for footnotes

*Allergen contents as „total food" as described in column ingredients according to gravimetric mixture
** Protein contents according to laboratory analysis of raw material (total nitrogen according to Kjeldahl with F=6,38 for milk protein and F=5,7 for wheat protein)
*** Protein contents according to literature values (approx. 80% casein and 10% β-latcoglobulin in total milk protein [36]; approx. 50% approx. β-Lactoglobulin in whey powder [31]; 8,7% gluten in wheat flour [37, 38])

Note: *The metrological traceability of temperature, mass and volume during production of the PT samples is ensured by DAkkS calibrated reference materials.*

2.1.1 Homogeneity

The **mixture homogeneity before bottling** was examined 8-fold by **microtracer analysis**. It is a standardized method that is part of the international GMP certification system for feed [14].
Before mixing dye coated iron particles of µm size are added to the sample and the number of particles is determined after homogenization in taken aliquots. The evaluation of the mixture homogeneity is based on the Poisson distribution using the chi-square test. A probability of ≥ 5 % is equivalent to a good homogeneous mixture and of ≥ 25% to an excellent mixture [14, 15].
The microtracer analysis of the present PT samples B and the spiking level sample showed a probability of 84% and 48%. Additionally particle number results were converted into concentrations, statistically evaluated according to normal distribution and compared to the standard deviation according to Horwitz. For the assessment HorRat values between 0,3 and 1,3 are to be accepted under repeat conditions (measurements within the laboratory) [17]. This gave a HorRat value 1,0 and 1,3. The HorRat value of >1,3 was accepted, because the probability was sufficient proof of homogeneity. The results of microtracer analysis are given in the documentation.

Homogeneity of bottled spiked sample B

Implementation of homogeneity tests
The homogeneity tests were carried out in cooperation with the laboratories of the specified test kit providers. Ten samples of the bottled spiked sample were chosen randomly by DLA, thereof 2 subsamples were weighed into previously randomly encoded sample containers, and then sent to the laboratories for analysis (exception: Morinaga ELISA II performed by DLA). The sample weights were made with a deviation of ± 10% from recommended sample weight of the test kit instructions and not communicated to the laboratories. After transmission of analysis results by the laboratories, the valid results were calculated on the basis of the exact weightings by DLA and the statistical calculation was carried out according to ISO 13528:2015 Annex B (possibly with Notes 1 and 2).

Valuation of homogeneity
The homogeneity is regarded as sufficient when the standard deviation between the samples Ss is ≤ 15% („heterogeneity standard deviation"). This criterion is fulfilled for sample B by all ELISA tests for β-lactoglobulin and casein (Immunolab, Morinaga and AgraQuant) and gluten (Immunolab, Morinaga and AgraQuant) (see page 7). Recommendations for repeatability standard deviations of ELISA and PCR methods are usually ≤ 25% [18, 19, 22, 23].

In case the criterion for sufficient homogeneity of the test items is not fulfilled the impact on the target standard deviation will be verified. If necessary the evaluation of results will be done considering the standard uncertainty of the assigned value by z'-scores (s. 3.6 and 3.8) [3].

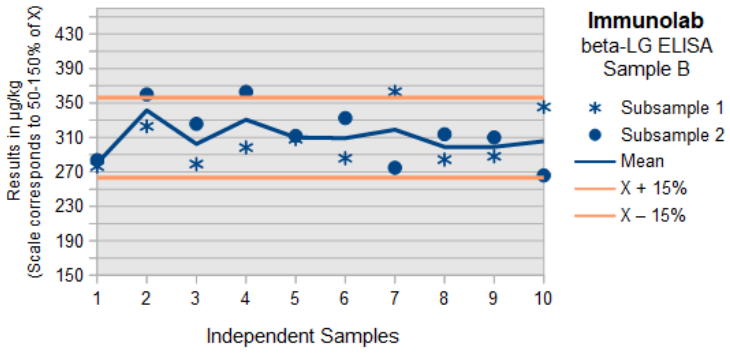
ELISA-Tests: Homogenität β-Lactoglobulin / Homogeneity β-Lactoglobulin

Immunolab β-Lactoglobulin ELISA

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: β-Lactoglobulin 310 ± 17 mg/kg

Sample B	Subsample 1 µg/kg	Subsample 2 µg/kg	Mean µg/kg
1	276	284	280
2	323	360	341
3	279	326	302
4	298	363	331
5	308	312	310
6	286	333	309
7	363	275	319
8	284	314	299
9	288	310	299
10	345	266	306

General average X	310	
SD of sample means Sx	17,3	5,6%
SD w ithin-samples Sw	25,2	8,1%
SD betw een-samples Ss	< 17,3	< 5,6%

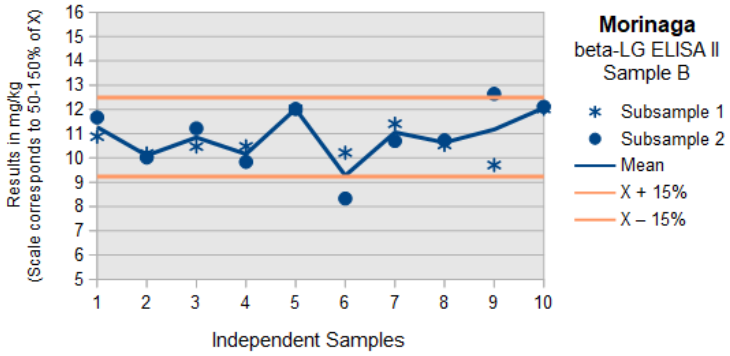


Morinaga Beta-lactoglobulin ELISA Kit II

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: β-Lactoglobulin 10,9 ± 0,9 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	10,9	11,7	11,3
2	10,2	10,0	10,1
3	10,5	11,2	10,8
4	10,5	9,8	10,2
5	12,0	12,0	12,0
6	10,2	8,3	9,3
7	11,4	10,7	11,1
8	10,5	10,7	10,6
9	9,7	12,6	11,2
10	12,0	12,1	12,1

General average X	10,9	
SD of sample means Sx	0,865	8,0%
SD w ithin-samples Sw	0,599	5,5%
SD betw een-samples Ss	0,754	6,9%



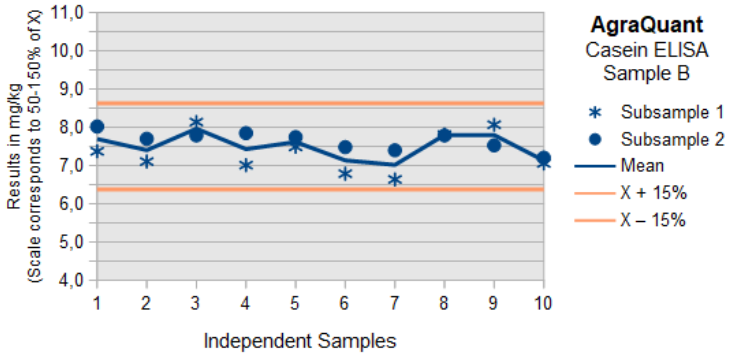
ELISA-Tests: Homogenität Casein / Homogeneity Casein

AgraQuant Casein ELISA

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Casein 7,50 ± 0,26 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	7,37	8,02	7,69
2	7,10	7,70	7,40
3	8,14	7,78	7,96
4	7,01	7,84	7,43
5	7,48	7,74	7,61
6	6,78	7,48	7,13
7	6,63	7,40	7,01
8	7,81	7,78	7,80
9	8,06	7,53	7,80
10	7,05	7,20	7,13

General average X	7,50	
SD of sample means Sx	0,327	4,4%
SD w ithin-samples Sw	0,276	3,7%
SD betw een-samples Ss	0,262	3,5%



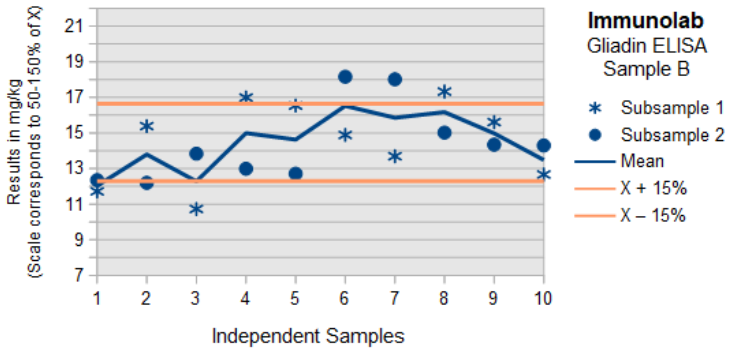
ELISA-Tests: Homogenität Gluten (Weizen) / Homogeneity Gluten (Wheat)

Immunolab Gliadin ELISA

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Gliadin 14,5 ± 1,1 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	11,7	12,4	12,0
2	15,4	12,2	13,8
3	10,7	13,8	12,3
4	17,0	13,0	15,0
5	16,5	12,7	14,6
6	14,9	18,2	16,5
7	13,7	18,0	15,9
8	17,3	15,0	16,2
9	15,6	14,3	15,0
10	12,7	14,3	13,5

General average X	14,5	
SD of sample means Sx	1,55	10,7%
SD within-samples Sw	1,50	10,4%
SD between-samples Ss	1,14	7,8%

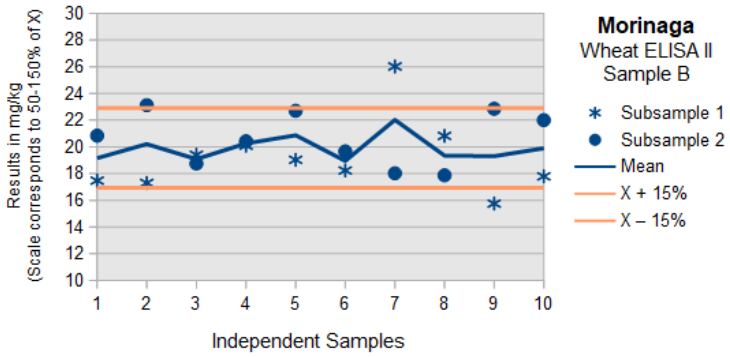


Morinaga Wheat ELISA Kit II

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Wheat protein 19,9 ± 0,9 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	17,5	20,8	19,2
2	17,3	23,1	20,2
3	19,4	18,8	19,1
4	20,1	20,4	20,3
5	19,0	22,7	20,9
6	18,2	19,7	18,9
7	26,0	18,0	22,0
8	20,8	17,9	19,3
9	15,8	22,9	19,3
10	17,8	22,0	19,9

General average X	19,9	
SD of sample means Sx	0,97	4,9%
SD within-samples Sw	2,25	11,3%
SD between-samples Ss	< 0,97	< 4,9%

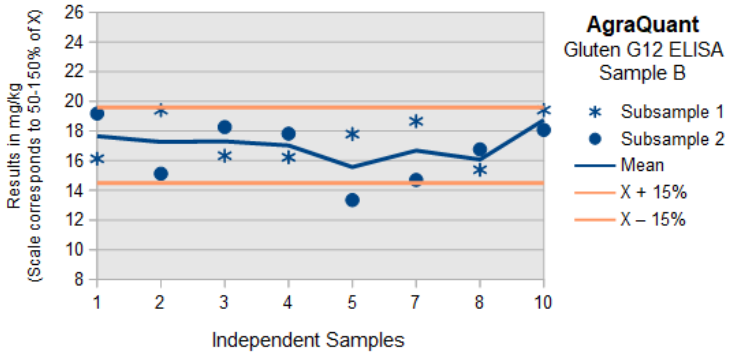


AgraQuant Gluten G12 ELISA

Sample weights: 1,0 g (0,9 – 1,1 g)
Number of replicates: 2
Overall result: Gluten 17,0 ± 1,0 mg/kg

Sample B	Subsample 1 mg/kg	Subsample 2 mg/kg	Mean mg/kg
1	16,1	19,2	17,7
2	19,4	15,1	17,3
3	16,3	18,3	17,3
4	16,2	17,8	17,0
5	17,8	13,3	15,6
7	18,7	14,7	16,7
8	15,4	16,8	16,1
10	19,4	18,1	18,7

General average X	17,0	
SD of sample means Sx	0,97	5,7%
SD within-samples Sw	1,51	8,9%
SD between-samples Ss	< 0,97	< 5,7%



2.1.2 Stability

A water activity (a_W) of < 0,5 is an important factor to ensure the stability of dry or dried products during storage. Optimum conditions for storage is the a_W value range of 0,15 - 0,3. In this range the lowest possible degradation rate is to be expected [16].

The experience with various DLA test materials showed good storage stability with respect to the durability of the sample (spoilage) and the content of the PT parameters for comparable food matrices and water activity (a_W value <0,5).
The a_W values of the EP food matrix samples were approx. 0,14 (20,7°C) and for the spiking level samples approx. 0,29 (18,9°). The stability of the sample material was thus ensured during the investigation period under the specified storage conditions.

2.2 Sample shipment and information to the test

The portions of test materials sample A, B and the spiking level sample were sent to every participating laboratory in the 17th week of 2021. The testing method was optional. The tests should be finished at 25th June 2021 the latest.

With the cover letter along with the sample shipment the following information was given to participants:

*There are two different samples A and B possibly containing the allergenic parameters **β-Lactoglobulin, Casein** and **Gluten** in the range of mg/kg in the matrix of hypoallergenic Infant Food with hydrolyzed Milk Protein. One of these samples and the "spiking level sample" were prepared adding the allergenic ingredients. The **"spiking level sample"** contains the allergens in a simple matrix in **similar amounts** without further processing and should be analysed like a normal sample.*

Please note the attached information on the proficiency test.
(see documentation, section 5.3 Information on the PT)

2.3 Submission of results

The participants submitted their results in standard forms, which have been handed out with the samples (by email).
On one hand the results given as positive/negative and on the other hand the indicated results of the allergenic ingredients e.g. total food item or protein in mg/kg were evaluated.
Queried and documented were the indicated results and details of the test methods like specificity, limit of quantifications, test kit manufacturer and hints about the procedure.
In case participants submitted several results for the same parameter obtained by different methods these results were evaluated with the same evaluation number with a letter as a suffix and indication of the related method.

20 out of 21 participants submitted at least one result. One participant

submitted no results.

3. Evaluation

Different ELISA-methods for the determination of allergens in foods are eventually using different antibodies, are usually calibrated with different reference materials and may utilize differing extraction methods. Among others this can induce different results of the content of the analyte [25, 26, 27, 28]. It is for this reason that we contrast the results of the present proficiency test with several assigned values.
Thereby it is possible to evaluate each single result in comparison to the mean of all results and/or in comparison to the mean of results obtained by a single method. For comparison the actually added amount is plotted in the figures of the results.

For quantitative results of the spiking level sample and the spiked sample recovery rates were calculated with respect to the known content of spiked allergens. The recovery rates were given for information only. No statistical evaluation was done. The recovery rates should exclusively give an estimation of the matrix- and/or processing influences.

ELISA- and PCR results were valuated qualitatively with respect to the percentages of positive and negative results, respectively. If there are ≥ 75 % positive or negative results, a consensus result is determined for each sample.

3.1 Consensus value from participants (assigned value)

The **robust mean** of the submitted results was used as assigned value (X_{pt}) („consensus value from participants") providing a normal distribution. The calculation was done according to algorithm A as described in annex C of ISO 13528 [3]. If there are < 12 quantitative results and an increased difference between robust mean and median, the **median** may be used as the assigned value (criterion: Δ median - rob. mean > 0,3 σ_{pt}) [3].
The condition is that the majority of the participants' results show a normal distribution or are distributed unimodal and symmetrically. To this end, an examination of the distribution is carried out, inter alia, using the kernel density estimate [3, 12].
In case there are indications for sources of higher variability such as a bimodal distribution of results, a cause analysis is performed. Frequently different analytical methods may cause an anomaly in results' distribution. If this is the case, separate evaluations with own assigned values (X_{pti}) are made whenever possible.

If possible, this is the standard procedure for the evaluation of methods for the quantitative determination of allergens:

i) ***Assigned value of all results - $X_{pt_{ALL}}$***
ii) ***Assigned value of single methods - $X_{pt_{METHOD\ i}}$***
with at least 5 quantitative results given.

Single results giving values outside the measuring range of the participating laboratory or given as „0" are not considered for statistical evaluation (e.g. results given as > 25 mg/kg and < 2,5 mg/kg, respectively) [3].

3.2 Robust standard deviation

For comparison to the target standard deviation σ_{pt} (standard deviation for proficiency assessment) a robust standard deviation (S*) was calculated. The calculation was done according to algorithm A as described in annex C of ISO 13528 [3].

The following robust standard deviations were considered:

i) ***Robust standard deviation of all results*** - S^x_{ALL}
ii) ***Robust standard deviation of single methods*** - $S^x_{METHOD\ i}$
with at least 5 quantitative results given.

3.3 Exclusion of results and outliers

Before statistical evaluation obvious blunders, such as those with incorrect units, decimal point errors, too few significant digits (valid digits) or results for another proficiency test item can be removed from the data set [2]. Even if a result e.g. with a factor >10 deviates significantly from the mean and has an influence on the robust statistics, a result of the statistical evaluation can be excluded [3].

All results should be given at least with 2 significant digits. Specifying 3 significant digits is usually sufficient.

Results obtained by different analytical methods causing an increased variability and/or a bi- or multimodal distribution of results, are treated separately or could be excluded in case of too few numbers of results. For this results are checked by kernel density estimation [3, 12].

Results are tested for outliers by the use of robust statistics (algorithm A): If a value deviates from the robust mean by more than 3 times the robust standard deviation, it can be classified as an outlier (see above) [3]. Due to the use of robust statistics outliers are not excluded, provided that no other reasons are present [3]. Detected outliers are only mentioned in the results section, if they have been excluded from the statistical evaluation.

3.4 Target standard deviation (for proficiency assessment)

The target standard deviation of the assigned value σ_{pt} (= standard deviation for proficiency assessment) can be determined according to the following methods.
In the present PT the target standard deviation was determined according to 3.4.3 value by perception.

3.4.1 General model (Horwitz)

Based on statistical characteristics obtained in numerous PTs for different parameters and methods Horwitz has derived a general model for estimating the reproducibility standard deviation σ_R [6]. Later the model was modified by Thompson for certain concentration ranges [10]. The reproducibility standard deviation σ_R can be applied as the relative target standard deviation σ_{pt} in % of the assigned values and calculated according to the following equations [3]. For this the assigned value X_{pt} is used for the concentration c.

Equations	**Range of concentrations**	**corresponds to**
$\sigma_R = 0{,}22c$	$c < 1{,}2 \times 10^{-7}$	< 120 µg/kg
$\sigma_R = 0{,}02c^{0{,}8495}$	$1{,}2 \times 10^{-7} \leq c \leq 0{,}138$	≥ 120 µg/kg
$\sigma_R = 0{,}01c^{0{,}5}$	$c > 0{,}138$	> 13,8 g/100g

with c = mass content of analyte (as relative size, e.g. 1 mg/kg = 1 ppm = 10^{-6} kg/kg)

The target standard deviation according to Horwitz is currently not achievable by ELISA or PCR-methods for values in the mg/kg range and was therefore not considered for evaluation.

3.4.2 Value by precision experiment

Using the reproducibility standard deviation σ_R and the repeatability standard deviation σ_r of a precision experiment (collaborative trial or proficiency test) the target standard deviation σ_{pt} can be derived considering the number of replicate measurements m of participants in the present PT [3]:

$$\sigma_{pt} = \sqrt{\sigma_R^2 - \sigma_r^2\left(m - 1/m\right)}$$

The relative repeatability standard deviations (RSD_r) and relative reproducibility standard deviations (RSD_R) given in table 2a (ELISA) and table 2b (PCR) were obtained in precision experiments by the indicated methods. The resulting target standard deviations σ_{pt} were calculated for a number of m = 2 replicate measurements. With a number of m = 1 replicate measurements the reproducibility standard deviation σ_R is identical to the target standard deviation σ_{pt}.

Table 2a: ELISA-Methods - Relative repeatability standard deviations (RSD_r) and relative reproducibility standard deviations (RSD_R) from precision experiments and resulting target standard deviations σ_{pt} [30-31]

Parameter	Matrix	Mean [mg/kg]	Recovery	rob RSD	RSD_r	RSD_R	σ_{pt}	Method / Literature
Peanut	Milk chocolate	173,7 33,8 5,9	87 % 85 % 59 %	- - -	8,8% 5,2% 7,8%	31% 20% 31%	30,4% 19,7% 30,5%	ELISA Manuf. A ASU 00.00-69
Peanut	Milk chocolate	215,7 40,1 10,1	108 % 100 % 101 %	- - -	5,9% 7,2% 7,3%	32% 14% 16%	31,7% 13,0% 15,1%	ELISA Manuf. B ASU 00.00-69
Peanut	Dark chocolate	148,2 30,9 5,7	74 % 77 % 57 %	- - -	6,0% 13% 6,1%	22% 25% 33%	21,6% 23,2% 32,7%	ELISA Manuf. A ASU 00.00-69
Hazelnut	Dark chocolate	16,3 7,56 3,73 1,62	81 % 76 % 75 % 81 %	- - - -	4,7% 8,9% 13% 15%	12% 15% 24% 33%	11,5% 13,6% 22,2% 31,2%	ELISA Manuf. A ASU 44.00-7
Hazelnut	Dark chocolate	21,3 10,7 4,69 2,37	106 % 107 % 94 % 119 %	- - - -	7,1% 11% 11% 9,3%	14% 19% 17% 17%	13,1% 17,3% 15,1% 16,4%	ELISA Manuf. B ASU 44.00-7

From the precision data of the official German ASU §64 methods the calculated relative target standard deviations are in the range of 12 – 33% for the ELISA methods and 18 – 37% for the PCR methods depending on the matrix, processing and concentration level of allergens (s. Tab. 2a and 2b).

The Working Group on Prolamin Analysis and Toxicity (WGPAT) coordinated a collaborative study with two commercial ELISA test kits for the determination of gluten using the monoclonal R5 antibody [24]. 12 food samples with gliadin in the range of 0 - 168 mg/kg were analyzed by 20 laboratories. Recovery rates ranged between 65 and 110%, relative repeatability deviations ranged from 13 - 25% (method 1) and 11 - 22% (method 2) while the relative reproducibility standard deviations ranged from 23 - 47% (method 1) and 25 - 33% (method 2). According to the authors both ELISA test kits fulfilled therefore the current validation criteria for ELISA methods [24].

The IRMM (Institute for Reference Materials and Measurements) performed an interlaboratory comparison for five different ELISA test kits for the quantification of peanut [27]. The mean values for two matrices were in the concentration range of 0,3 - 16,1 mg/kg and 1,2 - 20,4 mg/kg, respectively. The lowest relative reproducibility standard deviations of the five test kits were for dark chocolate in the range of 20 - 42% and for cookies in the range of 23 – 61%.

Table 2b: PCR-Methods - Relative repeatability standard deviations (RSD_r) and relative reproducibility standard deviations (RSD_R) from precision experiments and resulting target standard deviations σ_{pt} [32-35]

Parameter	**Matrix**	**Mean [mg/kg]**	**Recov-ery**	**rob RSD**	**RSD_r**	**RSD_R**	**σ_{pt}**	**Method / Literature**
Soya	Wheat flour Maize flour	107 145	107 % 145 %	63 % 34 %	- -	31 % 24 %	- -	rt-PCR ASU 16.01-9
Soya flour	Boiled saus-age (100°C, 60 min)	114,1 64,4	114 % 161 %	-	14,7% 27,7%	22,2% 41,4%	19,6% 36,5%	rt-PCR ASU 08.00-65
Soya flour	Sausage, autoclaved	33,1	33 %	-	21,5%	30,8	26,8%	rt-PCR ASU 08.00-65
Soya flour	Boiled saus-age (100°C, 60 min)	82,0 39,6 19,6 9,3	82 % 99 % 98 % 93 %	-	17,3% 22,9% 22,9% 31,1%	24,1% 31,8% 24,0% 30,2%	20,8% 27,4% 17,7% -	rt-PCR ASU 08.00-59
Wheat + Rye	Boiled saus-age (100°C, 60 min)	96,1	120 %	-	21,3%	35,4%	32,0%	rt-PCR ASU 08.00-66
Wheat + Rye	Sausage, autoclaved	74,9	11,0 %	-	24,6%	32,7%	27,7%	rt-PCR ASU 08.00-66

3.4.3 Value by perception

The target standard deviation for proficiency assessment can be set at a value that corresponds to the level of performance that the coordinator would wish laboratories to be able to achieve [3].
Criteria for the level of performance of analytical methods for the quantitative determination of allergens in foods were recently elaborated e.g. by the Ministry of Health and Welfare (MHLW) in Japan [22], by the working group 12 „Food Allergens" of the technical committee CEN/TC 275 [19-21], by an international "Food Allergen Working Group" under the advice of the AOAC Presidential Task Force on Food Allergens [23] and by the Codex Alimentarius Committee (CAC/GL 74-2010) [18].

Some of the relevant ELISA and PCR validation criteria of the mentioned panels are listed in tables 3 and 4, respectively.

Table 3: ELISA-Validation

Literature [18-24]	**Recovery rate**	**Repeatability standard deviation**	**Reproducibility standard deviation**
MHLW 2006	50 - 150%		≤ 25%
CEN 2009		≤ 20%	
AOAC 2010	50 - 150%	6,9 - 34,4% (a)	19,5 - 57,2% (a)
CAC 2010	70 - 120%	≤ 25%	≤ 35%

(a) = Example from an hypothetical proficiency scheme in the range of 0,5 - 5 mg/kg

Table 4: PCR-Validation

Literature [18]	**Recovery rate**	**Repeatability standard deviation**	**Reproducibility standard deviation**
CAC 2010	± 25% (a)	≤ 25%	≤ 35%

(a) = Trueness / Richtigkeit

Based on the currently achievable level of performance of ELISA and PCR methods for the quantitative determination of allergens in foods, which could be deduced from the data of precision experiments and from validation criteria, we set a relative target standard deviation σ_{pt} of 25%.
This target standard deviation was applied for the statistical evaluation of the results by z-score or if necessary by z´-Score and was used for all assigned values mentioned in 3.1.

3.5 z-Score

To assess the results of the participants the z-score is used. It indicates about which multiple of the target standard deviation (σ_{pt}) the result (x_i) of the participant is deviating from the assigned value (X_{pt}) [3].
Participants' z-scores are derived from:

$$z_i = \frac{\left(x_i - x_{pt}\right)}{\sigma_{pt}}$$

The requirements for the analytical performance are generally considered as fulfilled if

$$-2 \leq z \leq 2 \ .$$

For information the z-scores below are calculated with a target standard deviation of 25%:

i) ***z-Score*** - z_{ALL} *(with respect to all methods)*
ii) ***z-Score*** - $z_{METHOD\ i}$ *(with respect to single methods)*

3.5.1 Warning and action signals

In accordance with the norm ISO 13528 it is recommended that a result that gives rise to a *z*-score above 3,0 or below −3,0, shall be considered to give an "action signal" [3]. Likewise, a *z*-score above 2,0 or below −2,0 shall be considered to give a "warning signal". A single "action signal", or "warning signal" in two successive PT-rounds, shall be taken as evidence that an anomaly has occurred which requires investigation.
An error or cause analysis can be carried out by checking the analysis process including understanding and implementation of the measurement by the staff, details of the measurement procedure, calibration of equipment and composition of reagents, transmission or calculation errors, trueness and precision and use of reference material. If necessary appropriate corrective measures should be applied [3].

In the figures of z-scores DLA gives the limits of warning and action signals as yellow and red lines respectively. According to ISO 13528 the signals are valid only in case of a number of ≥ 10 results [3].

3.6 z'-Score

The z'-score can be used for the valuation of the results of the participants, in cases the standard uncertainty has to be considered (s. 3.8). The z'-score represents the relation of the deviation of the result (x_i) of the participant from the respective consensus value to the square root of quadrat sum of the target standard deviation (σ_{pt}) and the standard uncertainty ($U_{(Xpt)}$) [3].

The calculation is performed by:

$$z'_i = \frac{x_i - x_{pt}}{\sqrt{\sigma_{pt}^2 + u_{(x_{pt})}^2}}$$

If carried out an evaluation of the results by means of z'score, we have defined below the expression in the denominator as a target standard deviation σ_{pt}'.

The requirements for the analytical performance are generally considered as fulfilled if

$$-2 \leq z' \leq 2 \ .$$

For warning and action signals see 3.5.1.

3.7 Quotient S*/σ_{pt}

Following the HorRat-value the results of a proficiency-test can be considered convincing, if the quotient of robust standard deviation S* and target standard deviation σ_{pt} does not exceed the value of 2.
A value > 2 means an insufficient precision, i.e. the analytical method is too variable, or the variation between the test participants is higher than estimated. Thus the comparability of the results is not given [3].

3.8 Standard uncertainty and traceability

Every assigned value has a standard uncertainty that depends on the analytical method, differences between the analytical methods used, the test material, the number of participating laboratories (P) and on other factors. The standard uncertainty ($U_{(Xpt)}$) for this PT is calculated as follows [3]:

$$u_{(x_{pt})} = 1{,}25 \times \frac{s^*}{\sqrt{p}}$$

If $U_{(Xpt)} \leq 0{,}3\ \sigma_{pt}$ the standard uncertainty of the assigned value needs not to be included in the interpretation of the results of the PT [3]. Values exceeding 0,3 imply, that the target standard deviation could be too low with respect to the standard uncertainty of the assigned value.

The traceability of the assigned value is ensured on the basis of the consensus value as a robust mean of the participant results.

3.9 Figures of assigned values

The assigned values and spiking levels are indicated as coloured lines in the figures of results. This allows the comparison of a single result with different possible target values like the spiked level, the robust mean of all results and the robust mean of a single method.

3.10 Recovery rates: Spiking

For the results of the spiking level sample and the spiked sample recovery rates were calculated with respect to the known content of added allergens. The related values of added allergens are given in 2.1 test material in table 1. As a range of acceptance RA for valuating participant's results the range of 50 - 150% for the recovery rates of allergen-ELISAs proposed by the AOAC was used [23]. For quantitative PCR or LC/MS determinations we use the same range of acceptance.
The corresponding z-scores were calculated according to 3.5 with the target standard deviation of 25% (see 3.4.3).

4. Results

All following tables are anonymized. With the delivering of the evaluation report the participants are informed about their individual evaluation number.
Evaluation was done separately for ELISA and PCR-techniques. The results were grouped according to the applied methods (e.g. test kits) and sorted chronologically according to the evaluation number of the participants.
The following result sections are structured equally for the allergenic components. First all results of ELISA or PCR methods for a certain parameter are reported for samples A and B (qualitative / possibly quantitative) and afterwards for the spiking level sample (quantitative). The recovery rates of results for the spiking level sample and the spiked sample A or B are reported then.

In the result chapter all quantitative results of the participants are displayed formatted to 3 decimal places. In the documentation, all results are given as they were transmitted by the participants.

To ensure the **comparability of quantitative results** DLA harmonized participants' results giving different specifications (e.g. as protein or as allergenic food) as far as possible.

β-Lactoglobulin-specific ELISA results given as **total milk protein** were converted to **β-lactoglobulin** using contents from the literature [36] (approx. 10 % in total milk protein, see S.5) (Morinaga ELISA Kit II).

Casein-specific ELISA results given as **total milk protein** were converted to **casein** using contents from the literature [36] (approx. 80 % in total milk protein, see S.5) (Morinaga ELISA Kit II).

Milk protein-specific ELISA results reported as **skimmed milk powder** have been converted into **total milk protein**. If available, the specifications of the relevant test kit manufacturer for the total milk protein content in skimmed milk powder were taken into account (Neogen Allergen Handbook: 35,1%).

Milk protein-specific ELISA results, which were given as the **sum of casein and β-lactoglobulin** (SensiSpec ELISA), have not been converted, but have been equated with the total milk protein.

In the present PT all gluten ELISA results were submitted as gluten, therefore no conversion was necessary.

Results were valuated qualitatively with respect to the percentages of positive and negative results, respectively. If there are ≥ 75 % positive or negative results, a consensus result is determined for each sample. Each participant result is valuated qualitatively with respect to the consensus value. The valuation was given as a percentage of results in agreement with the consensus values.

When there are at least 5 quantitative results for all methods or for single methods a statistical evaluation was done.

In cases when a statistical evaluation of the quantitative values was done the result table was given as indicated below:

Evaluation number	Result	Result	z-Score Xpt_{ALL}	z-Score $Xpt_{M\,i}$	Method	Remarks
	pos/neg	[mg/kg]				

The statistical evaluation of results for each parameter was calculated in cases where at least 50% results were positive and at least 5 quantitative values were given:

Characteristics	**All Results** *[mg/kg]*	**Method i** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$	$X_{pt_{METHOD\ i}}$
Number of results		
Number of outliers		
Mean		
Median		
Robust mean (X_{pt})		
Robust standard deviation (S*)		
Target data°:		
Target standard deviation σ_{pt} *or* σ_{pt}'		
lower limit of target range ($X_{pt} - 2\sigma_{pt}$) or ($X_{pt} - 2\sigma_{pt}'$)°		
upper limit of target range ($X_{pt} + 2\sigma_{pt}$) or ($X_{pt} + 2\sigma_{pt}'$)°		
Quotient S*/σ_{pt} *or* S*/σ_{pt}'		
Standard uncertainty $U_{(Xpt)}$		
Number of results in target range		
Percent in target range		

° Target range calculated using z-score or z'-score

After that the recovery rates of the results for the spiking level sample and the spiked sample are reported. The number of results within the range of acceptance of 50-150% is given.

4.1 Proficiency Test Milk

4.1.1 ELISA Results: β-Lactoglobulin

Qualitative valuation of results: Samples A and B

Evaluation number	Sample A	Sample A	Sample B	Sample B	Qualitative Valuation	Method	Remarks
	pos/neg	[mg/kg]	pos/neg	[mg/kg]	Agreement with consensus value		
8	positive	1,41	positive	5,52	1/1 (100%)	AQ	
10	negative	<0,01	positive	0,34	1/1 (100%)	AQ	
11	-		positive	0,35	1/1 (100%)	AQ	
16	positive	0,80	positive	7,70	1/1 (100%)	IN	
4	positive	3,75	positive	13,3	1/1 (100%)	MI-II	result converted °
9	positive	0,86	positive	11,0	1/1 (100%)	MI-II	
7	positive	7,30	positive	33,5	1/1 (100%)	RS-C	
12	negative	<5	positive	26,7	1/1 (100%)	RS-C	
19	-		positive	29,0	1/1 (100%)	RS-C	
2	positive	0,57	positive	3,64	1/1 (100%)	RS-F	
13	positive	0,35	positive	12,2	1/1 (100%)	RS-F	
14	negative	< 0,167	positive	14,3	1/1 (100%)	RS-F	
15	negative	< 0,5	positive	14,0	1/1 (100%)	RS-F	
18	positive	0,58	positive	12,1	1/1 (100%)	RS-F	

° calculation p.20

	Sample A		Sample B	
Number positive	8		14	
Number negative	4		0	
Percent positive	67		100	
Percent negative	33		0	
Consensus value	none		positive	

Methods:
AQ = AgraQuant, RomerLabs
IN = INgezim, Ingenasa
MI-II = Morinaga Institute ELISA Kit II
RS-C = Ridascreen® competitive, R-Biopharm
RS-F= Ridascreen® Fast, R-Biopharm

Comments:
The consensus values for sample B are in qualitative agreement with the spiking of sample B.
For sample A, no consensus value of ≥ 75% positive or negative results was obtained. The sample matrix contains hydrolyzed milk proteins, which are probably the cause of the positive ELISA results.

Quantitative Valuation ELISA: Sample A

Due to the heterogeneity of the results, even when using the same ELISA methods, and due the small number of results from the same test kits, no quantitative evaluation of the results was carried out.

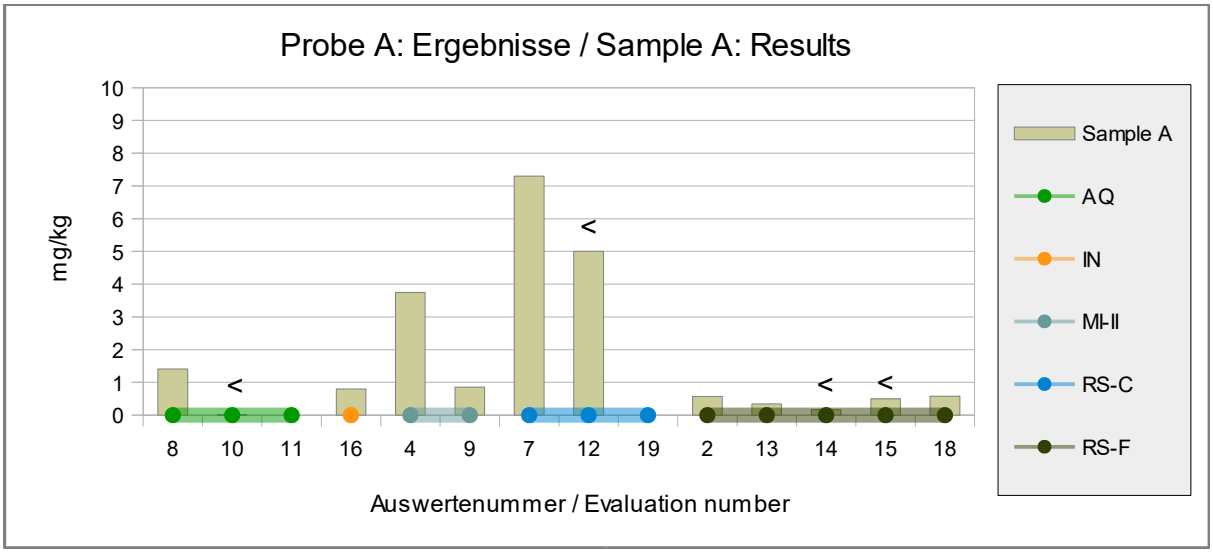


Abb./Fig. 1: ELISA Results β-Lactoblobulin
round symbols = Applied methods (see legend)

Quantitative valuation of ELISA-results: Sample B

Evaluation number	β-Lactoglo-bulin	z-Score Xpt_{ALL}	z-Score Xpt_{RS-F}	Method	Remarks
	[mg/kg]				
8	5,52			AQ	
10	0,34			AQ	
11	0,35			AQ	
16	7,70	-1,3		IN	
4	13,3	0,66		MI-II	result converted °
9	11,0	-0,14		MI-II	
7	33,5			RS-C	
12	26,7			RS-C	
19	29,0			RS-C	
2	3,64	-2,7	-2,8	RS-F	
13	12,2	0,28	0,08	RS-F	
14	14,3	1,0	0,78	RS-F	
15	14,0	0,92	0,68	RS-F	
18	12,1	0,25	0,05	RS-F	

° calculation p.20

Methods:

AQ = AgraQuant, RomerLabs
IN = INgezim, Ingenasa
MI-II = Morinaga Institute ELISA Kit II
RS-C = Ridascreen® competitive, R-Biopharm
RS-F= Ridascreen® Fast, R-Biopharm

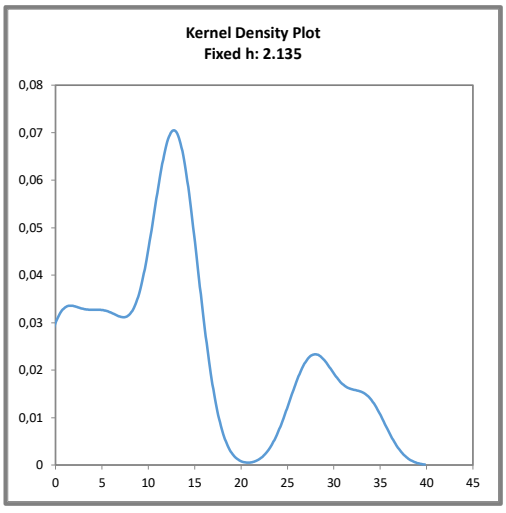


Abb. / Fig. 2:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σ_{pt} von Xpt_{ALL})

Kernel density plot of all ELISA results (with h = 0,75 x σ_{pt} of Xpt_{ALL})

Comments:
The kernel density estimation shows nearly a symmetrical distribution of results for the main peak ("Peak 11"). The results below and above the central peak can be assigned to methods AQ (<7 mg/kg) and RS-C (>20 mg/kg).

Characteristics: Quantitative evaluation ELISA β-Lactoglobulin

Sample B

Statistic Data	**Meth. "Peak 11"** *[mg/kg]*	**Method RS-F** *[mg/kg]*
Assigned value (X_{pt})	$\boldsymbol{X_{pt_{PEAK\ 11}}}$	$\boldsymbol{X_{pt_{RS\text{-}F}}}$
Number of results	8	5
Number of outliers	0	0
Mean	11,0	11,3
Median	12,2	12,2
Robust Mean (X_{pt})	**11,4**	**12,0**
Robust standard deviation (S*)	**3,25**	**3,23**
Target range:		
Target standard deviation σ_{pt}	**2,85**	**2,99**
lower limit of target range	**5,69**	**5,98**
upper limit of target range	**17,1**	**17,9**
Quotient S/σ_{pt}*	*1,1*	*1,1*
Standard uncertainty $U_{(Xpt)}$	*1,43*	*1,81*
Results in the target range	*7*	*4*
Percent in the target range	*88*	*80*

Methods:
PEAK 11 = INgezim (Ingenasa), ELISA Kit II (Morinaga Inst.), Ridascreen® Fast (R-Biopharm)
RS-F = Ridascreen® Fast (R-Biopharm)

Comments to the statistical characteristics and assigned values:

The kernel density estimation showed a method-dependent distribution of the results, so that no evaluation of all methods together was carried out. The results of the "Peak 11" methods were jointly evaluated (see Fig. 2).

The evaluation of the results of the "Peak 11" methods as well as the results of method RS-F showed a normal to low variability of results. The quotients S/σ_{pt} were well below 2,0. The robust standard deviations were in the range of established values for the repeatability and reproducibility standard deviation of the applied methods (see 3.4.2 value by precision experiments and 3.4.3 value by perception). The comparability of results is given. This conclusion is limited for the evaluation across the methods, because there were only a few results for some methods.*

The robust means of the evaluations were 50% ($X_{Peak\ 11}$) and 53% ($X_{RS\text{-}F}$) of the spiking level of β-lactoglobulin to sample B and were in the range of the recommendations for the applied methods, whereby it must taken into account that low levels were detected in sample A as well (s. 3.4.3 and p.32 "Recovery rates with z-scores ELISA for β-lactoglobulin ").

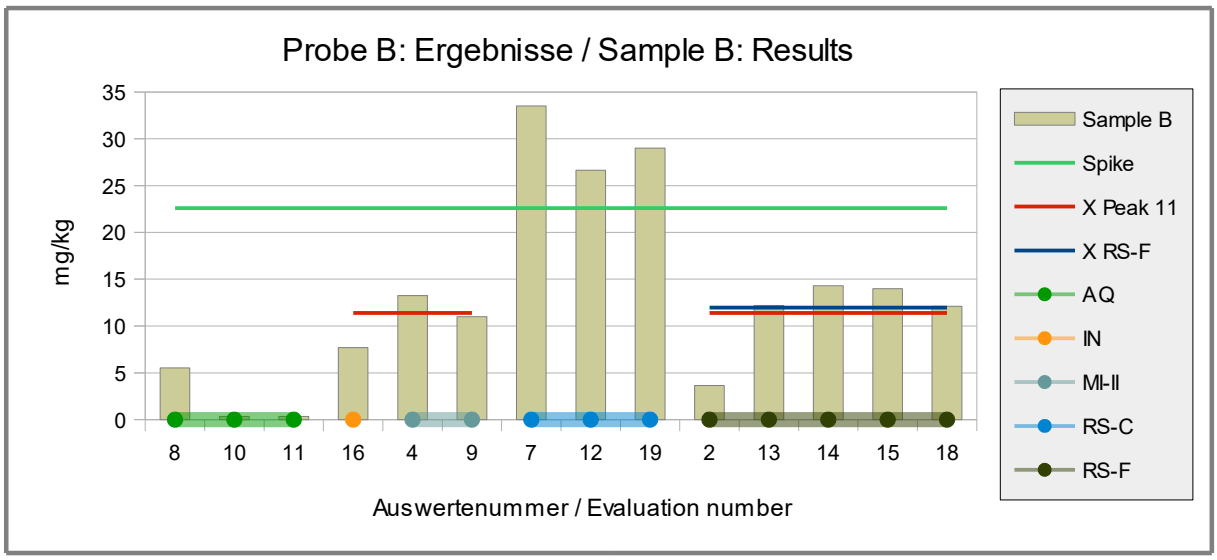


Abb./Fig. 3: ELISA Results β-Lactoblobulin
green line = Spiking level (Spike)
red line = Assigned value robust mean all results
blue line = Assigned value robust mean method RS-F
round symbols = Applied methods (see legend)

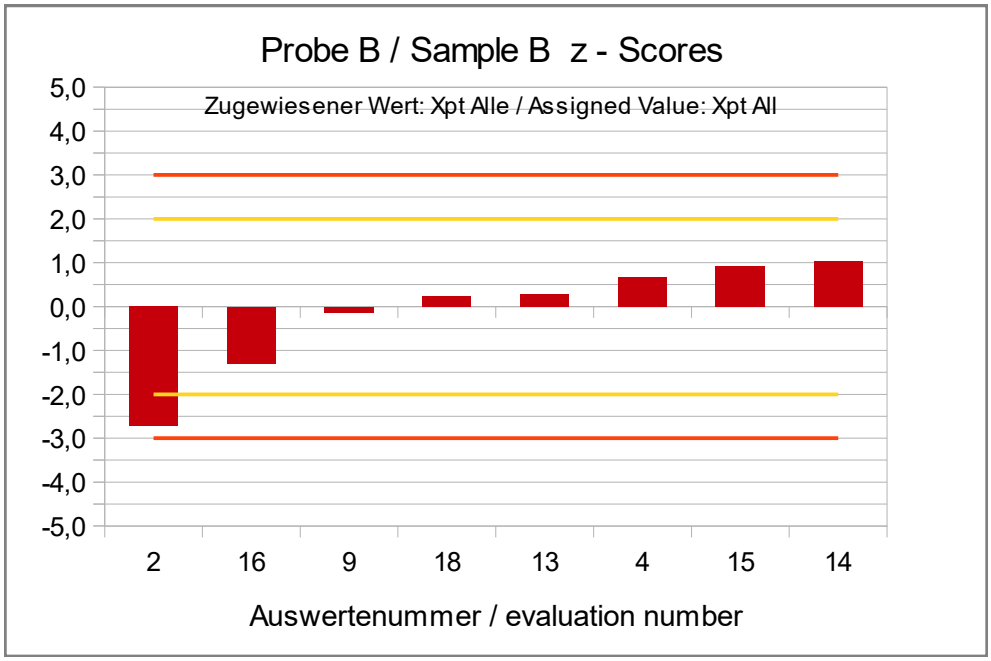


Abb./Fig. 4:
z-Scores ELISA Results β-Lactoblobulin
Assigned value robust mean of "Peak 11" results

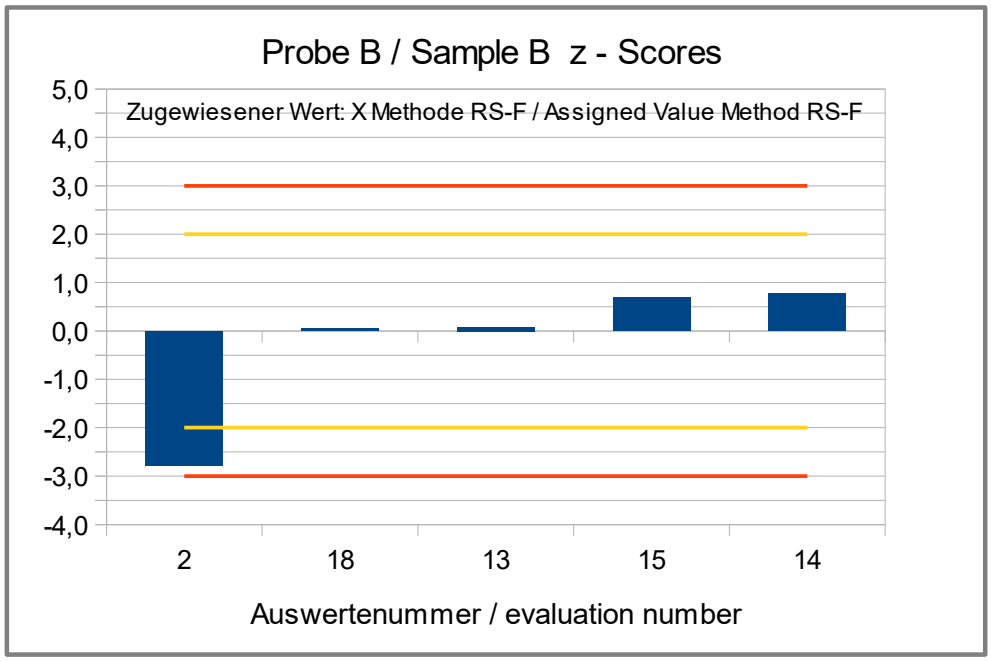


Abb./Fig. 5:
z-Scores ELISA Results β-Lactoblobulin, Assigned value robust mean of results method RS-F (R-Biopharm, Ridascreen Fast)

Quantitative valuation of ELISA: Spiking Level Sample

Evaluation number	β-Lactoglo-bulin	z-Score Xpt_{ALL}	z-Score $Xpt_{RS\text{-}F}$	Method	Remarks
	[mg/kg]				
8	9,22	-1,5		AQ	
10	3,95	-2,9		AQ	
11	> 0,4			AQ	
16	15,8	0,33		IN	
4	17,0	0,65		MI-II	result converted °
9	14,0	-0,17		MI-II	
7	13,6	-0,28		RS-C	
12	10,1	-1,2		RS-C	
19	12,0	-0,71		RS-C	
2	15,9	0,35	-0,47	RS-F	
13	16,6	0,55	-0,32	RS-F	
14	18,0	0,93	-0,01	RS-F	
15	21,0	1,8	0,66	RS-F	
18	18,7	1,1	0,15	RS-F	

° calculation p.20

Methods:

AQ = AgraQuant, RomerLabs

IN = INgezim, Ingenasa

MI-II = Morinaga Institute ELISA Kit II

RS-C = Ridascreen® competitive, R-Biopharm

RS-F= Ridascreen® Fast, R-Biopharm

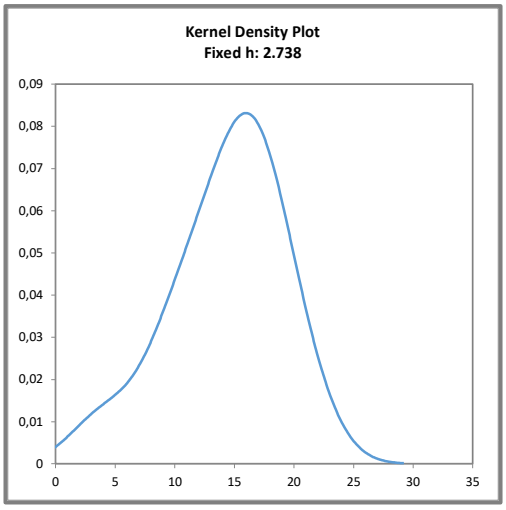


Abb. / Fig. 6:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σpt von Xpt_{ALL})

Kernel density plot of all ELISA results (with h = 0,75 x σpt of Xpt_{ALL})

Comment:
The kernel density estimation shows almost a symmetrical distribution of results with a small shoulder < 7 mg/kg.

Characteristics: Quantitative evaluation ELISA β-Lactoblobulin

Spiking Level Sample

Statistic Data	**All Results** *[mg/kg]*	**Method RS-F** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$	$X_{pt_{METHOD\ RS\text{-}F}}$
Number of results	13	5
Number of outliers	0	0
Mean	14,3	18,0
Median	15,8	18,0
Robust Mean (X_{pt})	**14,6**	**18,0**
Robust standard deviation (S*)	**4,43**	**2,26**
Target range:		
Target standard deviation σ_{pt}	**3,65**	**4,51**
lower limit of target range	**7,30**	**9,02**
upper limit of target range	**21,9**	**27,1**
Quotient S/σ_{pt}*	*1,2*	*0,50*
Standard uncertainty $U_{(Xpt)}$	*1,54*	*1,26*
Results in the target range	*12*	*5*
Percent in the target range	*92*	*100*

Methoden:
RS-F = R-Biopharm, Ridascreen® Fast

Comments to the statistical characteristics and assigned values:

The kernel density estimation showed a distribution without clear method-dependent differences.

The evaluation of the results of all methods as well as the results of method RS-F showed a normal and low variability, respectively. The quotients S/σ_{pt} were below 2,0 and below 1,0. The robust standard deviations are in the upper range of established values for the repeatability and reproducibility standard deviation of the applied methods (see 3.4.2 value by precision experiments and 3.4.3 value by perception). The comparability of results is given.*
This conclusion is limited for the evaluation across the methods, because there were only a few results for some methods.

The robust means of the evaluations were 48% (X_{ALL}) and 59% ($X_{RS\text{-}F}$) of the spiking level of β-lactoglobulin to the spiking level sample and were in the range of the recommendations for the applied methods (s. 3.4.3 and p.32 "Recovery rates ELISA for β-lactoglobulin").

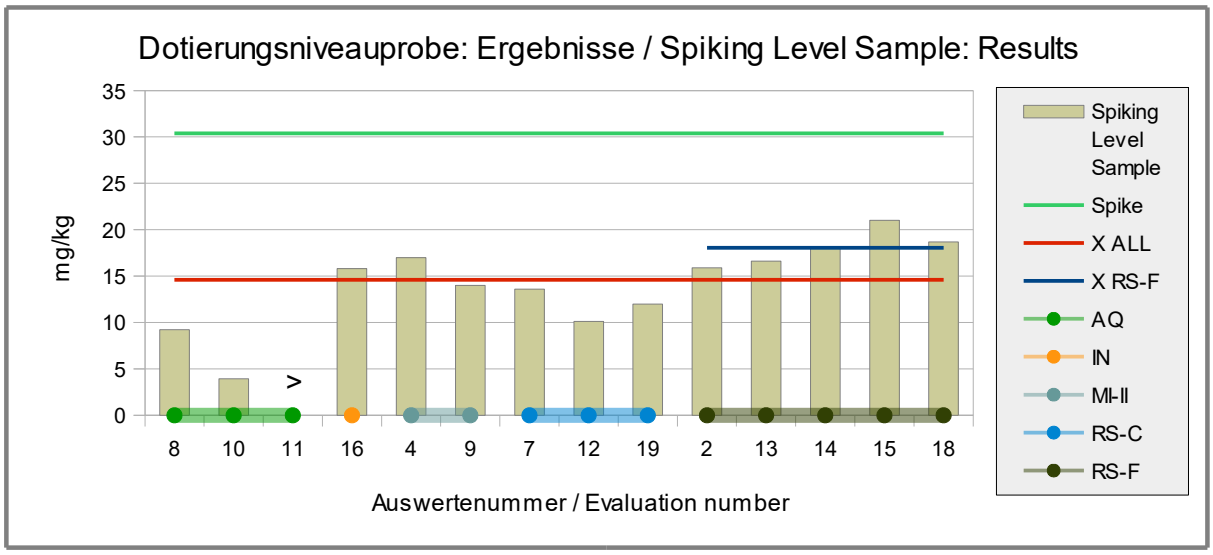


Abb./Fig. 7: ELISA Results β-Lactoglobulin
green line = Spiking level (Spike)
red line = Assigned value robust mean all results
blue line = Assigned value robust mean method RS-F
round symbols = Applied methods (see legend)

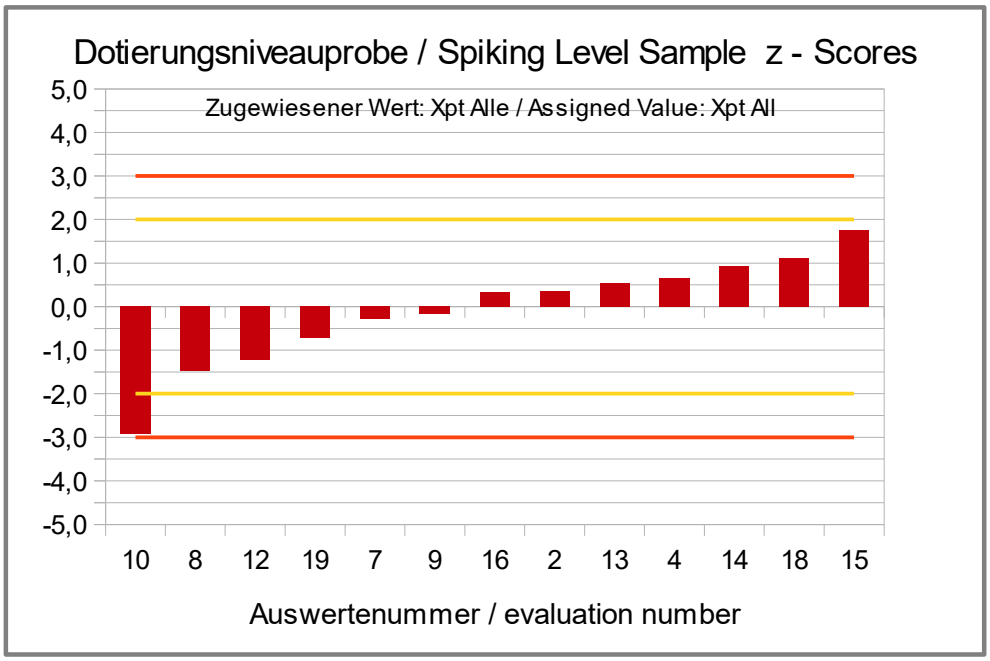


Abb./Fig. 8:
z-Scores ELISA Results β-Lactoglobulin
Assigned value robust mean of all results

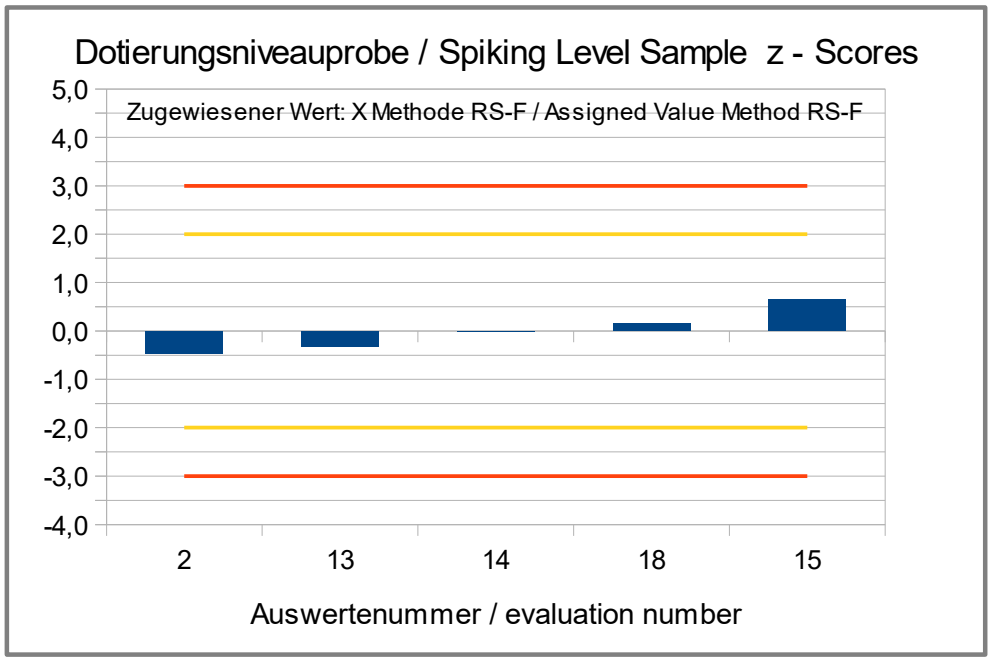


Abb./Fig. 9:
z-Scores ELISA Results β-Lactoglobulin Assigned value robust mean of results method RS-F (R-Biopharm, Ridascreen Fast)

Recovery Rates with z-Scores ELISA for β-lactoglobulin: Spiking Level Sample and Sample B

Evaluation number	Spiking Level Sample	Recovery rate*		Sample B-A (difference)	Recovery rate*		Method	Remarks
	[mg/kg]	[%]	[Z_{RR}]	[mg/kg]	[%]	[Z_{RR}]		
8	9,22	30	-2,8	4,11	18	-3,3	AQ	
10	3,95	13	-3,5	0,34	1,5	-3,9	AQ	
11	> 0,4			0,35	1,5	-3,9	AQ	
16	15,8	**52**	-1,9	6,90	31	-2,8	IN	
4	17,0	**56**	-1,8	9,51	42	-2,3	MI-II	results converted °
9	14,0	46	-2,2	10,1	45	-2,2	MI-II	
7	13,6	45	-2,2	26,2	**116**	0,64	RS-C	
12	10,1	33	-2,7	26,7	**118**	0,72	RS-C	
19	12,0	39	-2,4	29,0	**128**	1,1	RS-C	
2	15,9	**52**	-1,9	3,07	14	-3,5	RS-F	
13	16,6	**55**	-1,8	11,9	**52**	-1,9	RS-F	
14	18,0	**59**	-1,6	14,3	**63**	-1,5	RS-F	
15	21,0	**69**	-1,2	14,0	**62**	-1,5	RS-F	
18	18,7	**62**	-1,5	11,5	**51**	-2,0	RS-F	

° calculation p.20

RA**	50-150 %	RA**	50-150 %
Number in RA	**7**	Number in RA	**7**
Percent in RA	**54**	Percent in RA	**50**

* Recovery rate 100% relative size: beta-Lactoglobulin, s. page 5

** Range of acceptance of AOAC for allergen ELISAS

Methods:

AQ = AgraQuant, RomerLabs

IN = INgezim, Ingenasa

MI-II = Morinaga Institute ELISA Kit II

RS-C = Ridascreen® competitive, R-Biopharm

RS-F= Ridascreen® Fast, R-Biopharm

Comment:

54% (7) of the participants obtained for the spiking level sample a recovery rate by ELISA in the range of the AOAC recommendation of 50-150%. For the calculation of the recovery rates of the spiked sample B, the β-lactoglobulin results of the food matrix sample A, if given, were subtracted from the results for sample B. Thus 50% (7) of the recovery rates for sample B were in the acceptance range of 50-150%.
The related z-scores are based on the target standard deviation of 25%.

Note to Method RS-C:

Native, intact β-lactoglobulin is under-estimated in the test kit compared to hydrolyzed β-lactoglobulin (more epitopes are available for antibody binding in hydrolyzed foods) (Test Kit Manual, R4901).

4.1.2 ELISA Results: Casein

Qualitative valuation of results: Samples A and B

Evaluation number	Sample A	Sample A	Sample B	Sample B	Qualitative Valuation	Method	Remarks
	pos/neg	[mg/kg]	pos/neg	[mg/kg]	Agreement with consensus value		
11	negative	<0,2	positive	4,9	2/2 (100%)	AQ	
17	negative	<1	positive	7,6	2/2 (100%)	AQ	
18	negative	0,46	positive	8,2	2/2 (100%)	AQ	
10	negative	<0,2	positive	3,84	2/2 (100%)	IL	
16a	negative	<0,3	positive	8,7	2/2 (100%)	IN	
8	negative		positive	12,01	2/2 (100%)	MI	result converted °
4	negative	<0,62	positive	16,68	2/2 (100%)	MI-II	result converted °
9	negative	<0,25	positive	13	2/2 (100%)	MI-II	
1	negative	<0.5	positive	2,5	2/2 (100%)	RS-F	
2	positive	2,7	positive	11,3	1/2 (50%)	RS-F	
3	negative		positive	3,2	2/2 (100%)	RS-F	
6	negative	<0.5	positive	4,6	2/2 (100%)	RS-F	
13	negative	<0,5	positive	5,3	2/2 (100%)	RS-F	
14	negative	< 2,5	positive	17	2/2 (100%)	RS-F	
15	negative	< 0,5	positive	2,6	2/2 (100%)	RS-F	
19	-		positive	8,6	1/1 (100%)	RS-F	
16b	negative	<0,2	positive	6,6	2/2 (100%)	SP	

° calculation p.20

	Sample A		Sample B	
Number positive	1		17	
Number negative	15		0	
Percent positive	6		100	
Percent negative	94		0	
Consensus value	negative		positive	

Methods:

AQ = AgraQuant, RomerLabs
IL = Immunolab
MI = Morinaga Institute ELISA
MI-II = Morinaga Institute ELISA Kit II
RS-F= Ridascreen® Fast, R-Biopharm
SP = SensiSpec ELISA Kit, Eurofins

Comments:
The consensus values are in qualitative agreement with the spiking of sample B.

Quantitative valuation of ELISA-results: Sample B

Note: Due to the heterogeneity of the quantitative results and the sometimes small number of results from individual methods, the statistical analysis was carried out for informational purposes only.

Evaluation number	Casein	z'-Score Xpt_{ALL}	z-Score Xpt_{RS-F}	Method	Remarks
	[mg/kg]				
11	4,90	-1,2		AQ	
17	7,60	-0,09		AQ	
18	8,20	0,16		AQ	
10	3,84	-1,6		IL	
16a	8,70	0,37		IN	
8	12,0	1,7		MI	result converted °
4	16,7	3,7		MI-II	result converted °
9	13,0	2,1		MI-II	
1	2,50	-2,2	-2,0	RS-F	
2	11,3	1,4	5,1	RS-F	
3	3,20	-1,9	-1,4	RS-F	
6	4,60	-1,3	-0,28	RS-F	
13	5,30	-1,0	0,28	RS-F	
14	17	3,8	9,7	RS-F	
15	2,60	-2,2	-1,9	RS-F	
19	8,60	0,33	2,9	RS-F	
16b	6,60	-0,50		SP	

° calculation p.20

Methods:

AQ = AgraQuant, RomerLabs
IL = Immunolab
MI = Morinaga Institute ELISA
MI-II = Morinaga Institute ELISA Kit II
RS-F= Ridascreen® Fast, R-Biopharm
SP = SensiSpec ELISA Kit, Eurofins

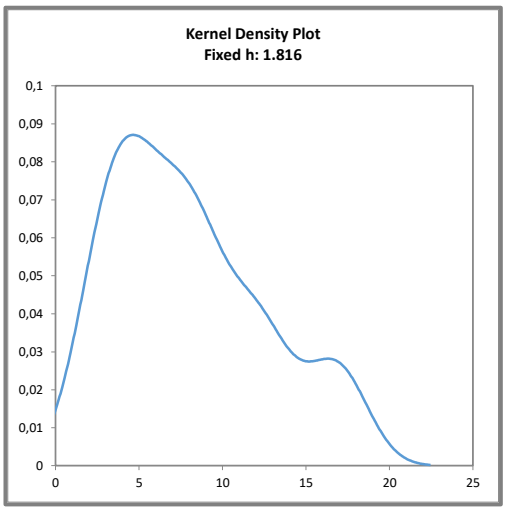


Abb. / Fig. 10:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σ_{pt} von Xpt_{ALL})

Kernel density plot of all ELISA results (with h = 0,75 x σ_{pt} of Xpt_{ALL})

Comments:
The kernel density estimation shows a broad distribution of results with a maximum at around 5 mg/kg and several shoulders up to a range of 20 mg/kg.

Characteristics: Quantitative evaluation ELISA Casein

Note: Due to the heterogeneity of the quantitative results and the sometimes small number of results from individual methods, the statistical analysis was carried out for informational purposes only.

Sample B

Statistic Data	**All Results** *[mg/kg]*	**Method RS-F** *[mg/kg]*
Assigned value (*Xpt*)	$X_{pt_{ALL}}$	$X_{pt_{METHOD\ RS\text{-}F}}$
Number of results	17	8
Number of outliers	0	0
Mean	8,04	6,89
Median (*Xpt*)⁺	7,60	**4,95**
Robust Mean (*Xpt*)⁺⁺	**7,81**	6,48
Robust standard deviation (S*)	**4,72**	**4,82**
Target range:		
Target standard deviation σ_{pt}'	**2,42**	**1,24**
lower limit of target range	**2,97**	**2,48**
upper limit of target range	**12,7**	**7,43**
Quotient S/σ_{pt}'*	*2,0*	*3,9*
Standard uncertainty U(Xpt)	*1,43*	*2,13*
Results in the target range	*12*	*5*
Percent in the target range	*71*	*63*

⁺ Assigned value (*Xpt*) for method RS-F: Median

⁺⁺ Assigned value (*Xpt*) for all results: Robust mean

Methods:
RS-F = R-Biopharm, Ridascreen® Fast

Comments to the statistical characteristics and assigned values:

The kernel density estimation shows a broad distribution of the results with possibly method-dependent differences. For method RS-F, ≥ 5 individual results were available, so that a separate evaluation was possible. Only 1-3 results were available for the other methods. For this reason, no separate evaluations could be carried out. Therefore, despite the wide distribution, only an informative evaluation of all methods was carried out. The resulting target range is not valid for the individual methods.

The distribution of the results of all methods showed an increased variability with a quotient S/σ_{pt} of > 2,0. Therefore, the z'-Score was used for the evaluation, taking into account the standard uncertainty. The distribution of the results of the method RS-F showed also an increased variability with a quotient S*/σ_{pt} of > 2,0. An evaluation using the z'-Score was dispensed with, as the target range would otherwise be inappropriately large.*

The robust mean and median of the evaluations were 50% (X_{ALL}) and 32% (X_{RS-F}) of the spiking level of casein to sample B and thus just within and below the range of the recommendations for the applied methods, respectively (s. 3.4.3 and p.42 "Recovery rates with z-scores ELISA for casein").

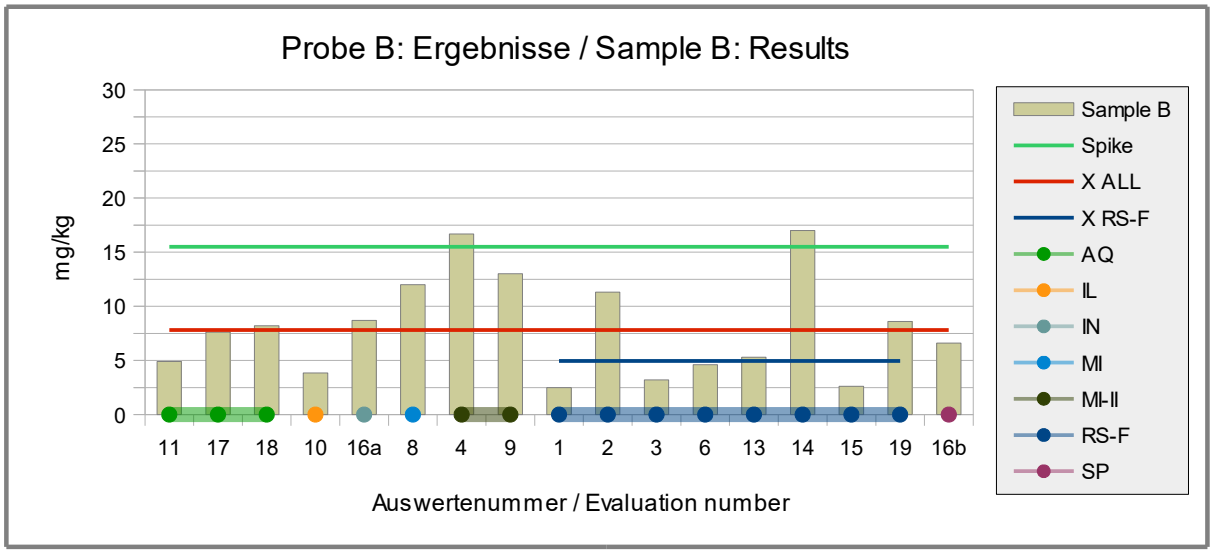


Abb./Fig. 11: ELISA Results Casein
green line = Spiking level (Spike)
red line = Assigned value robust mean all results
blue line = Assigned value robust mean method RS-F
round symbols = Applied methods (see legend)

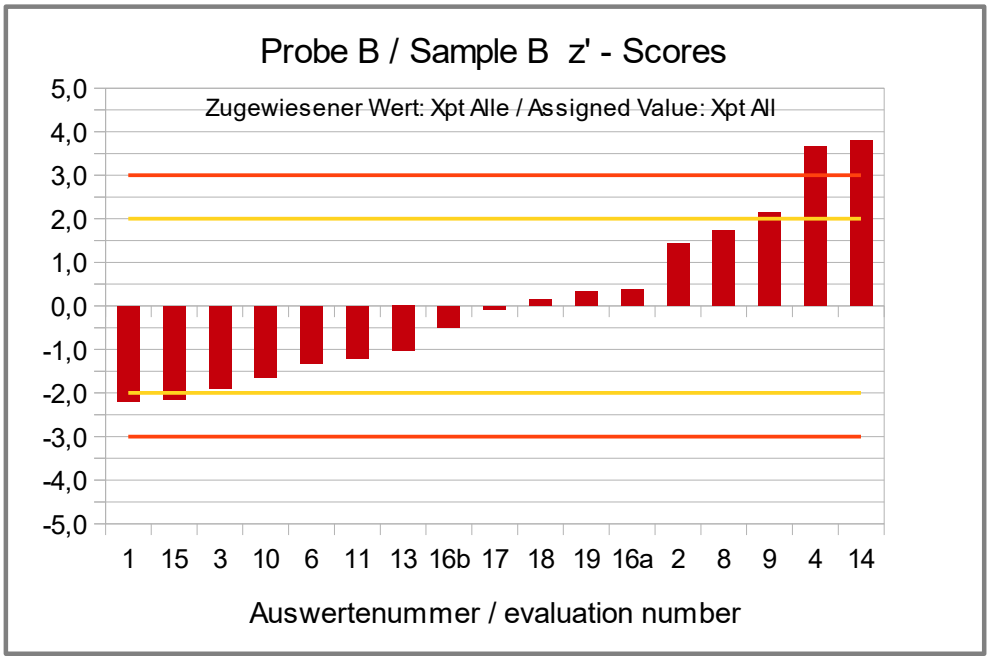


Abb./Fig. 12:
z'-Scores ELISA Results Casein

Assigned value robust mean of all results

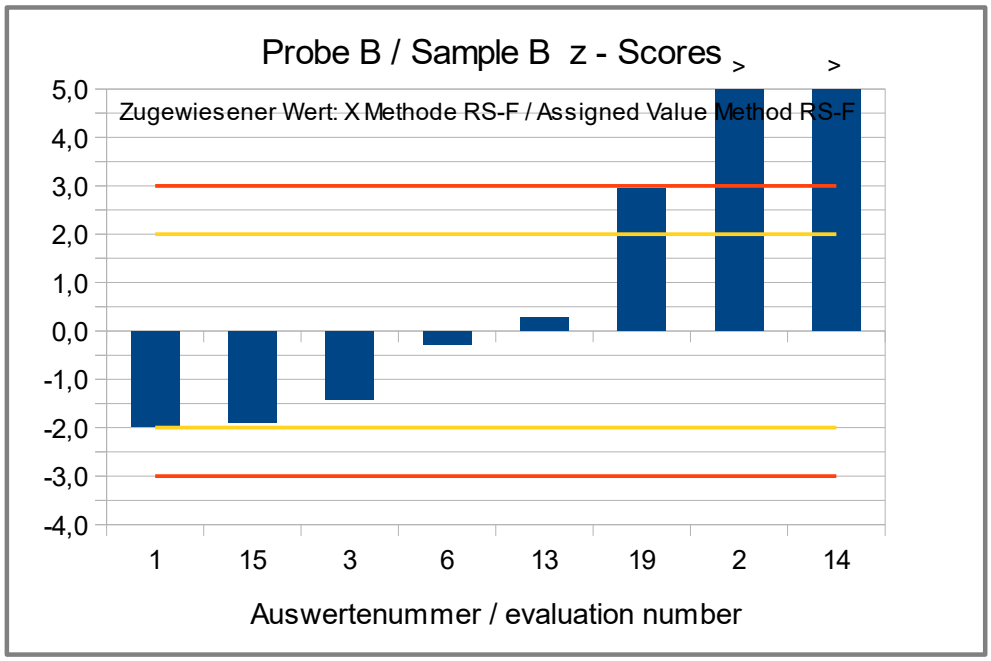


Abb./Fig. 13:
z-Scores ELISA Results Casein, Assigned value median of results method RS-F (R-Biopharm, Ridascreen Fast)

Quantitative valuation of ELISA: Spiking Level Sample

Evaluation number	Casein	z-Score Xpt_{ALL}	z-Score Xpt_{RS-F}	Method	Remarks
	[mg/kg]				
11	9,4	-2,3		AQ	
17	22,1	-0,09		AQ	
18	18,6	-0,70		AQ	
10	29,0	1,1		IL	
16a	21,9	-0,12		IN	
8	15,8	-1,2		MI	result converted °
4	21,1	-0,26		MI-II	result converted °
9	13,0	-1,7		MI-II	
1	22,5	-0,01	-0,25	RS-F	
2	24,0	0,25	0,00	RS-F	
3	38,7	2,9	2,5	RS-F	
6	>13.5			RS-F	
13	23,2	0,11	-0,13	RS-F	
14	24,0	0,25	0,00	RS-F	
15	22,0	-0,10	-0,33	RS-F	
19	49,0	4,7	4,2	RS-F	
16b	26,3	0,66		SP	

° calculation p.20

Methods:

AQ = AgraQuant, RomerLabs

IL = Immunolab

MI = Morinaga Institute ELISA

MI-II = Morinaga Institute ELISA Kit II

RS-F= Ridascreen® Fast, R-Biopharm

SP = SensiSpec ELISA Kit, Eurofins

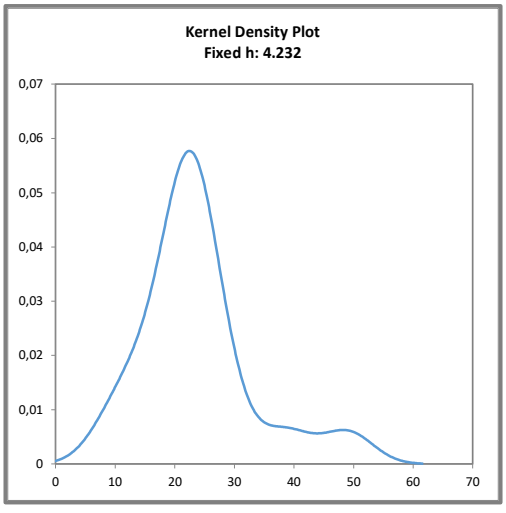


Abb. / Fig. 14:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σ_{pt} von Xpt_{ALL})

Kernel density plot of all ELISA results (with h = 0,75 x σ_{pt} of Xpt_{ALL})

Comment:
The kernel density estimation shows a main peak with nearly a symmetrical distribution of results with a slight shoulder at approx. 10 mg/kg and smaller peaks > 30 mg/kg.

Characteristics: Quantitative evaluation ELISA Casein

Spiking Level Sample

Statistic Data	**All Results** *[mg/kg]*	**Method RS-F** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$	$X_{pt_{METHOD\ RS\text{-}F}}$
Number of results	16	7
Number of outliers	–	–
Mean	23,8	29,1
Median (X_{pt})⁺	22,3	**24,0**
Robust Mean (X_{pt})⁺⁺	**22,6**	27,8
Robust standard deviation (S*)	**6,56**	**6,64**
Target range:		
Target standard deviation σ_{pt}	**5,64**	**6,00**
lower limit of target range	**11,3**	**12,0**
upper limit of target range	**33,9**	**36,0**
Quotient S/σ_{pt}'*	*1,2*	*1,1*
Standard uncertainty $U_{(Xpt)}$	*2,05*	*3,14*
Results in the target range	*13*	*5*
Percent in the target range	*81*	*71*

⁺ Assigned value (*Xpt*) for method RS-F: Median

⁺⁺ Assigned value (*Xpt*) for all results: Robust mean

Methoden:
RS-F = R-Biopharm, Ridascreen® Fast

Comments to the statistical characteristics and assigned values:

The kernel density estimation showed an almost symmetrical distribution.

The evaluations of all methods and of method RS-F showed normal variabilities of results. The quotients S/σ_{pt} were below 2,0. The robust standard deviations are in the range of established values for the reproducibility standard deviation of the applied methods (see 3.4.2 value by precision experiments and 3.4.3 value by perception). The comparability of results is given. This conclusion is limited for the evaluation across the methods, because there were only a few results for some methods.*

The robust means of the evaluations were 147% (X_{ALL}) and 156% ($X_{RS\text{-}F}$) of the spiking level of casein to the spiking level sample and thus within and slightly above the range of the recommendations for the applied methods, respectively (s. 3.4.3 and p.42 "Recovery rates ELISA for casein").

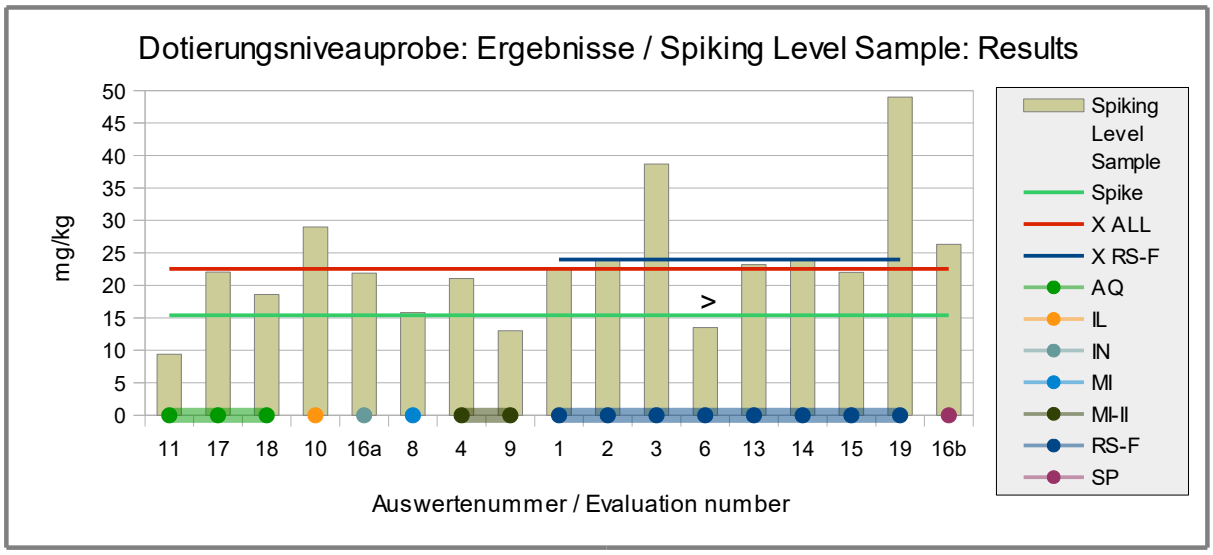


Abb./Fig. 15: ELISA Results Casein
green line = Spiking level (Spike)
red line = Assigned value robust mean all results
blue line = Assigned value robust mean method RS-F
round symbols = Applied methods (see legend)

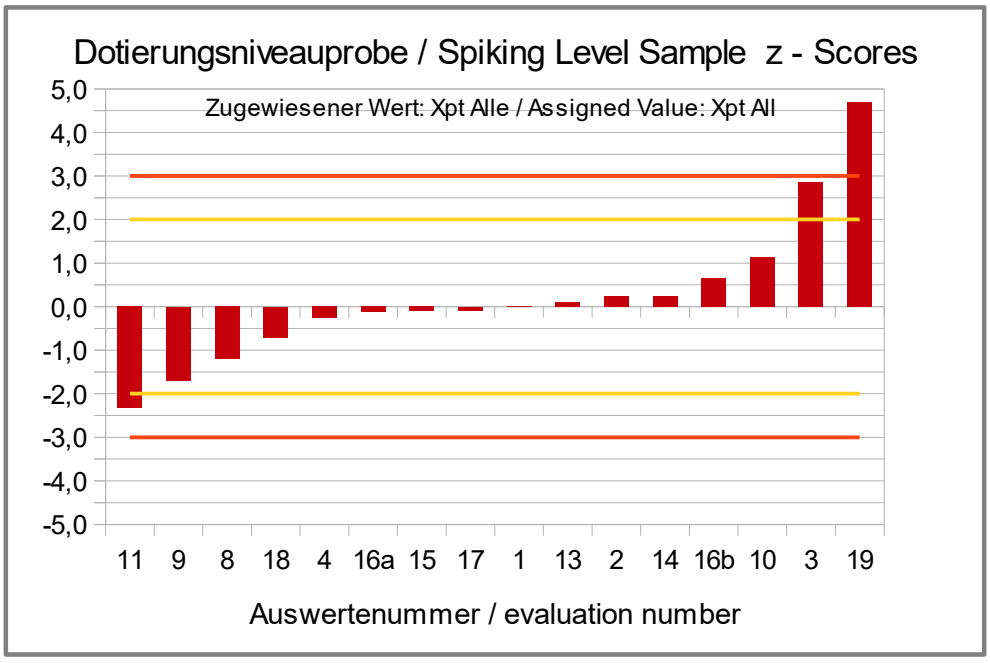


Abb./Fig. 16:
z-Scores ELISA Results Casein
Assigned value robust mean of all results

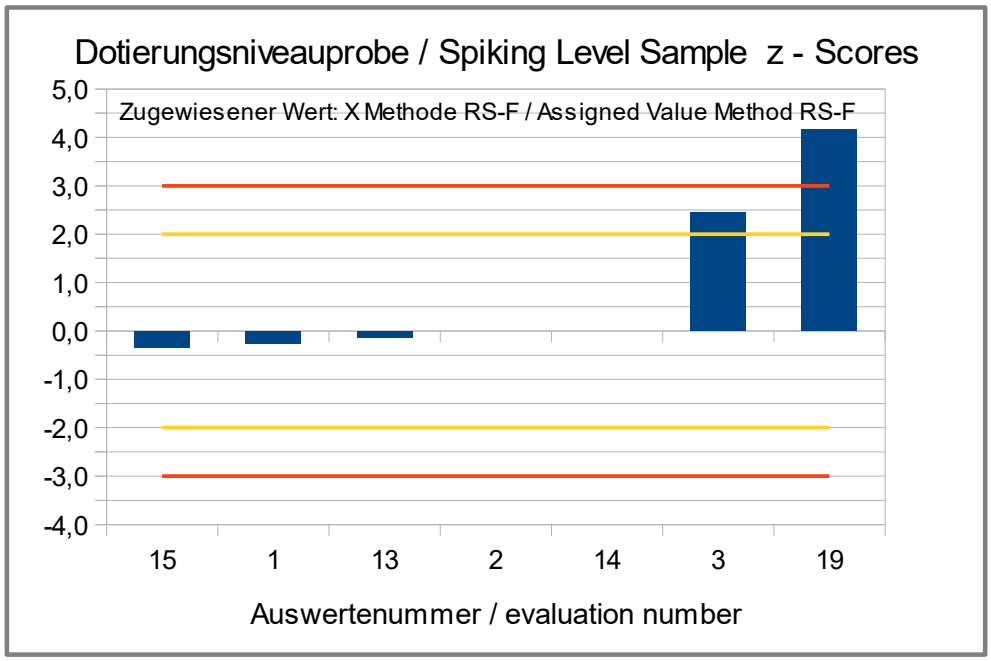


Abb./Fig. 17:
z-Scores ELISA Results Casein, Assigned value median of results method RS-F (R-Biopharm, Ridascreen Fast)

Recovery Rates with z-Scores ELISA for casein: Spiking Level Sample and Sample B

Evaluation number	Spiking Level Sample	Recovery rate*		Sample B	Recovery rate*		Method	Remarks
	[mg/kg]	[%]	[Z_{RR}]	[mg/kg]	[%]	[Z_{RR}]		
11	9,40	61	-1,6	4,90	32	-2,7	AQ	
17	22,1	**143**	1,7	7,60	49	-2,0	AQ	
18	18,6	**121**	0,83	8,20	**53**	-1,9	AQ	
10	29,0	188	3,5	3,84	25	-3,0	IL	
16a	21,9	**142**	1,7	8,70	**56**	-1,8	IN	
8	15,8	**103**	0,10	12,0	**77**	-0,90	MI	result converted °
4	21,1	**137**	1,5	16,7	**108**	0,30	MI-II	result converted °
9	13,0	**84**	-0,62	13,0	**84**	-0,65	MI-II	
1	22,5	**146**	1,8	2,50	16	-3,4	RS-F	
2	24,0	156	2,2	11,3	**73**	-1,1	RS-F	
3	38,7	251	6,1	3,20	21	-3,2	RS-F	
6	>13.5			4,60	30	-2,8	RS-F	
13	23,2	151	2,0	5,30	34	-2,6	RS-F	
14	24,0	156	2,2	17,0	**110**	0,39	RS-F	
15	22,0	**143**	1,7	2,60	17	-3,3	RS-F	
19	49,0	318	8,7	8,60	**55**	-1,8	RS-F	
16b	26,3	171	2,8	6,60	43	-2,3	SP	

° calculation p.20

RA**	50-150 %	RA**	50-150 %
Number in RA	9	Number in RA	9
Percent in RA	56	Percent in RA	53

* Recovery rate 100% relative size: Casein, s. page 5

** Range of acceptance of AOAC for allergen ELISAS

Methods:

AQ = AgraQuant, RomerLabs

IL = Immunolab

MI = Morinaga Institute ELISA

MI-II = Morinaga Institute ELISA Kit II

RS-F= Ridascreen® Fast, R-Biopharm

SP = SensiSpec ELISA Kit, Eurofins

Comment:

56% (9) of the participants obtained for the spiking level sample a recovery rate by ELISA methods within the range of the AOAC-recommendation of 50-150%. For the spiked food matrix sample B 53% (9) of the obtained recovery rates were within the recommended range.

With one exception, all recovery rates for sample B with hydrolyzed milk proteins in the matrix were lower than for the spiking level sample (matrix potato powder). The recovery rate was reduced by more than half in 12 of 16 result sets. Peptides of milk proteins may lead to a partial masking of antibodies of the test methods.

The related z-scores are based on the target standard deviation of 25%.

4.1.3 ELISA Results: Milk (as total milk protein)

Qualitative valuation of results: Samples A and B

Evaluation number	Sample A	Sample A	Sample B	Sample B	Qualitative Valuation	Method	Remarks
	pos/neg	[mg/kg]	pos/neg	[mg/kg]	Agreement with consensus value		
2	positive	8,2	positive	218	2/2 (100%)	RS-F	
3	positive	7,1	positive	16,3	2/2 (100%)	RS-F	
6	positive	6,5	positive	>67,5	2/2 (100%)	RS-F	
13	positive	9,8	positive	36,3	2/2 (100%)	RS-F	
16	-	-	positive	226	2/2 (100%)	RS-F	
9	positive	0,91	positive	12,0	2/2 (100%)	SP	
20	positive	0,80	positive	15,6	2/2 (100%)	SP	
5	negative	< LOQ	positive	11,0	1/2 (50%)	VT	
10	negative	<0,88	positive	4,39	1/2 (50%)	VT	result converted °

° calculation p.20

	Sample A		Sample B	
Number positive	6		9	
Number negative	2		0	
Percent positive	75		100	
Percent negative	25		0	
Consensus value	positive		positive	

Methods:

RS-F= Ridascreen® Fast, R-Biopharm

SP = SensiSpec ELISA Kit, Eurofins

VT = Veratox, Neogen

Comments:

The consensus value for sample B is in qualitative agreement with the spiking of sample B. For sample A, a consensus value of ≥ 75% positive results was also obtained. The sample matrix contains hydrolyzed milk proteins, which are probably the cause of the positive ELISA results.

Quantitative valuation of ELISA: Sample A and Sample B

Due to the heterogeneity of the results and the small number of results from the same test kits, no quantitative evaluation of the results was carried out.

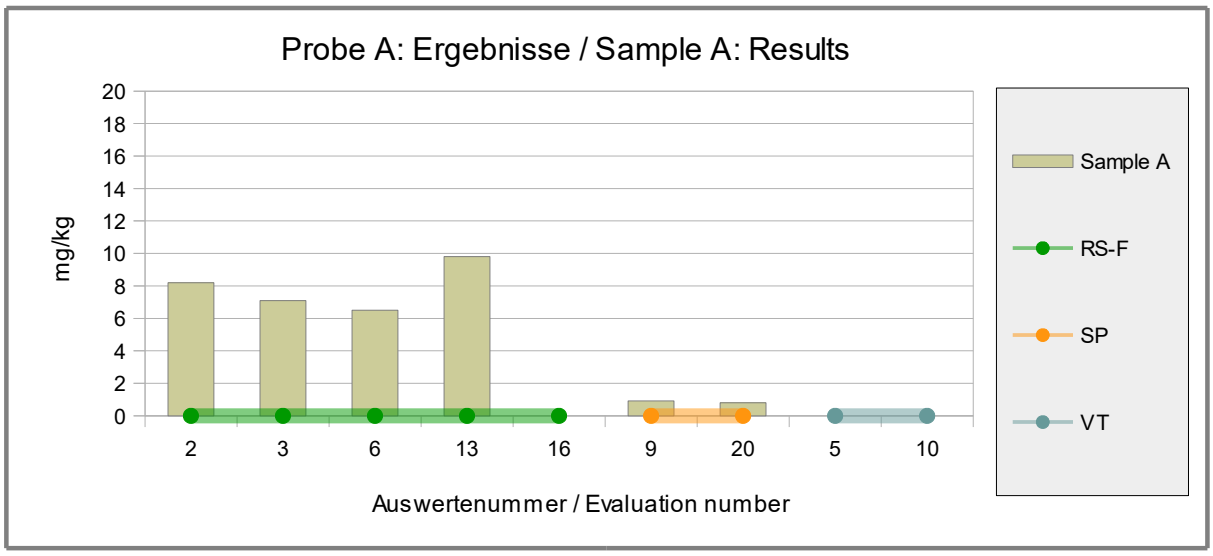


Abb./Fig. 18: ELISA Results Milk (as total milk protein)
round symbols = Applied methods (see legend)

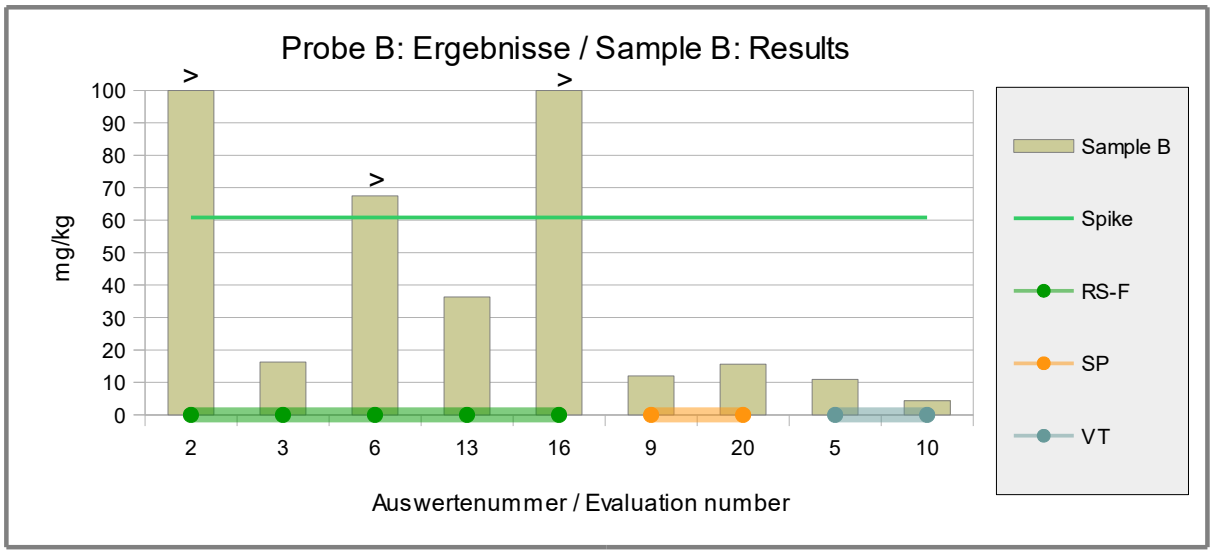


Abb./Fig. 19: ELISA Results Milk (as total milk protein)
round symbols = Applied methods (see legend)

Quantitative valuation of ELISA: Spiking Level Sample

Due to the heterogeneity of the results and the small number of results from the same test kits, no quantitative evaluation of the results was carried out.

Evaluation number	Total Milk protein	Total Milk protein	z-Score Xpt_{ALL}	Method	Remarks
	pos/neg	[mg/kg]			
2	positive	203		RS-F	
3	positive	317		RS-F	
6	positive	>67,5		RS-F	
13	positive	43,2		RS-F	
16	positive	345		RS-F	
9	positive	39,0		SP	
20	positive	51,8		SP	
5	positive	65,0		VT	
10	positive	47,4		VT	result converted °

° calculation p.20

Number positive	9
Number negative	0
Percent positive	100
Percent negative	0
Consensus value	positive

Methods:
RS-F= Ridascreen® Fast, R-Biopharm
SP = SensiSpec ELISA Kit, Eurofins
VT = Veratox, Neogen

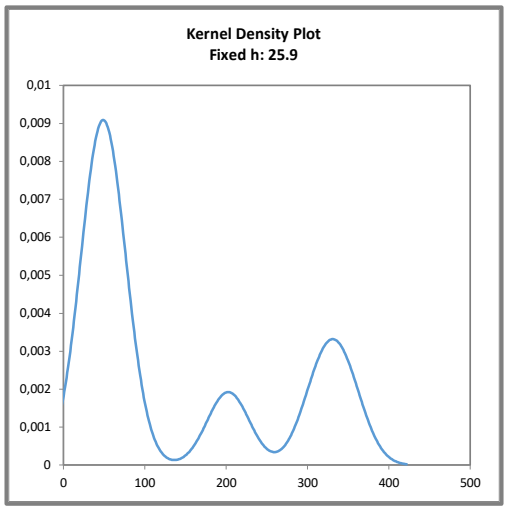


Abb. / Fig. 20:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σ_{pt} von Xpt_{ALL})

Kernel density plot of all ELISA results (with h = 0,75 x σ_{pt} of Xpt_{ALL})

Comment:
The kernel density estimation shows a main peak with two smaller side peaks.

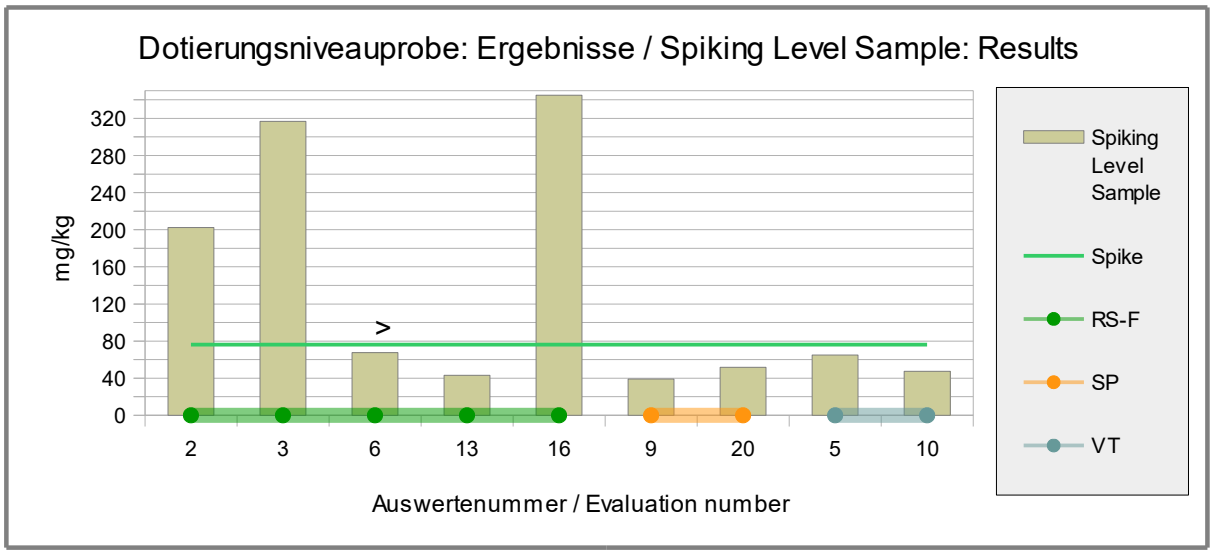


Abb./Fig. 21: ELISA Results Milk (as total milk protein)
round symbols = Applied methods (see legend)

Recovery Rates with z-Scores ELISA for milk: Spiking Level Sample and Sample B

Evaluation number	Spiking Level Sample	Recovery rate*		Sample B-A (difference)	Recovery rate*		Method	Remarks
	[mg/kg]	[%]	[Z_{RR}]	[mg/kg]	[%]	[Z_{RR}]		
2	203	266	6,6	210	345	10	RS-F	
3	317	416	13	9,20	15	-3,4	RS-F	
6	>67,5						RS-F	
13	43,2	**57**	-1,7	26,5	44	-2,3	RS-F	
16	345	453	14	226	372	11	RS-F	
9	39,0	**51**	-2,0	11,1	18	-3,3	SP	
20	51,8	**68**	-1,3	14,8	24	-3,0	SP	
5	65,0	**85**	-0,59	11,0	18	-3,3	VT	
10	47,4	**62**	-1,5	4,39	7,2	-3,7	VT	results converted °

° calculation p.20

RA**	50-150 %	RA**	50-150 %
Number in RA	**5**	Number in RA	**0**
Percent in RA	**63**	Percent in RA	**0**

Methods:
RS-F= Ridascreen® Fast, R-Biopharm
SP = SensiSpec ELISA Kit, Eurofins
VT = Veratox, Neogen

* Recovery rate 100% relative size: Milkproteins, total, s. page 5

** Range of acceptance of AOAC for allergen ELISAS

Comments:
63% (5) of the participants obtained for the spiking level sample a recovery rate by ELISA in the range of the AOAC requirement of 50-150%. For the calculation of the recovery rates of the spiked sample B, the milk protein results of the food matrix sample A, if given, were subtracted from the results for sample B. Thus none of the recovery rates for sample B were in the acceptance range of 50-150%.
The related z-scores are based on the target standard deviation of 25%.

It should be noted that the milk protein composition of the PT samples does not correspond to the natural ratio of casein to whey protein. The whey protein content is increased (s. page 5). Depending on the specificity of the methods used, this can lead to a changed response for total milk protein.

4.2 Proficiency Test Wheat (Gluten)

4.2.1 ELISA Results: Gluten

Qualitative valuation of results: Samples A and B

Evaluation number	Sample A	Sample A	Sample B	Sample B	Qualitativee Valuation	Method	Remarks
	pos/neg	[mg/kg]	pos/neg	[mg/kg]	Agreement with consensus value		
20	negative	0	positive	26,0	2/2 (100%)	IL	
1	negative	<3,0	positive	19,8	2/2 (100%)	RS	
2	negative	<5	positive	17,5	2/2 (100%)	RS	
3	negative		positive	164	2/2 (100%)	RS	
4	negative	<5	positive	12,4	2/2 (100%)	RS	
5	negative	< LOQ	positive	35,8	2/2 (100%)	RS	
6	negative	<10	positive	22,1	2/2 (100%)	RS	
7	negative	<5	positive	26,9	2/2 (100%)	RS	
8	negative		positive	25,1	2/2 (100%)	RS	
9a	negative	<5	positive	18,0	2/2 (100%)	RS	
12	negative	<5	positive	21,9	2/2 (100%)	RS	
13	negative	<6,6	positive	22,1	2/2 (100%)	RS	
14	negative	<5,0	positive	19,6	2/2 (100%)	RS	
15	negative	<5	positive	22,0	2/2 (100%)	RS	
16a	negative	<5,0	positive	21,0	2/2 (100%)	RS	
17	negative	<5	positive	24,0	2/2 (100%)	RS	
18	negative		positive	18,9	2/2 (100%)	RS	
19	-		positive	26,0	1/1 (100%)	RS	
9b	negative	<5	positive	20,0	2/2 (100%)	SP-H	
9c	negative	<3,12	positive	20,0	2/2 (100%)	SP-R5	
16b	negative	<5,0	positive	17,1	2/2 (100%)	SP-R5	
10	negative	<3,0	positive	16,6	2/2 (100%)	VT-R5	

	Sample A		Sample B	
Number positivee	0		22	
Number negativee	21		0	
Percent positivee	0		100	
Percent negativee	100		0	
Consensus value	negative		positive	

Methods:
IL = Immunolab
RS = Ridascreen®, R-Biopharm
SP-H = SensiSpec INgezim Hydrolysed Gluten, Eurofins
SP-R5 = SensiSpec INgezim Gluten R5, Eurofins
VT-R5 = Veratox, Neogen

Comments:
The consensus values are in qualitative agreement with the spiking of sample B.

Quantitative valuation of results: Sample B

Evaluation number	Gluten	z-Score Xpt_{ALL}	z-Score Xpt_{RS}	Method	Remarks
	[mg/kg]				
20	26,0	0,81		IL	
1	19,8	-0,34	-0,44	RS	
2	17,5	-0,76	-0,85	RS	
3	164	26,2	25,4	RS	
4	12,4	-1,7	-1,8	RS	
5	35,8	2,6	2,4	RS	
6	22,1	0,09	-0,03	RS	
7	26,9	0,97	0,84	RS	
8	25,1	0,64	0,51	RS	
9a	18,0	-0,67	-0,76	RS	
12	21,9	0,04	-0,07	RS	
13	22,1	0,09	-0,03	RS	
14	19,6	-0,38	-0,48	RS	
15	22,0	0,07	-0,04	RS	
16a	21,0	-0,12	-0,22	RS	
17	24,0	0,43	0,31	RS	
18	18,9	-0,50	-0,60	RS	
19	26,0	0,81	0,67	RS	
9b	20,0	-0,30		SP-H	
9c	20,0	-0,30		SP-R5	
16b	17,1	-0,84		SP-R5	
10	16,6	-0,93		VT-R5	

Methoden:

IL = Immunolab
RS = Ridascreen®, R-Biopharm
SP-H = SensiSpec INgezim Hydrolysed Gluten, Eurofins
SP-R5 = SensiSpec INgezim Gluten R5, Eurofins
VT-R5 = Veratox, Neogen

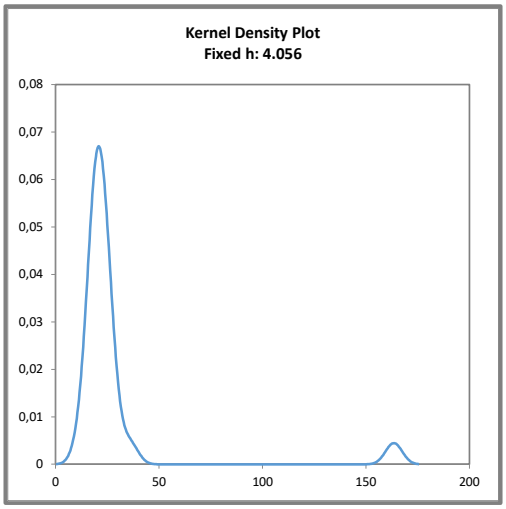


Abb. / Fig. 22:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σpt von Xpt_{ALL})

Kernel density plot of all ELISA results (with h = 0,75 x σpt of Xpt_{ALL})

Comments:
The kernel density estimation shows nearly a symmetrical distribution of results with a small side peak at 164 mg/kg, due to a single value outside the target range.

Characteristics: Quantitative evaluation ELISA Gluten

Sample B

Statistic Data	**All Results** *[mg/kg]*	**Method RS** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$	$X_{pt_{METHOD\ RS}}$
Number of results	22	17
Number of outliers	-	-
Mean	28,0	30,4
Median	21,4	22,0
Robust Mean (X_{pt})	**21,6**	**22,3**
Robust standard deviation (S*)	**4,34**	**4,46**
Target range:		
Target standard deviation σ_{pt}	**5,41**	**5,56**
lower limit of target range	**10,8**	**11,1**
upper limit of target range	**32,5**	**33,4**
Quotient S/σ_{pt}*	*0,80*	*0,80*
Standard uncertainty $U_{(Xpt)}$	*1,16*	*1,35*
Results in the target range	*20*	*15*
Percent in the target range	*91*	*88*

Methods:
RS = R-Biopharm, Ridascreen®

Comments to the statistical characteristics and assigned values:

The kernel density estimation showed nearly a symmetrical distribution.

The evaluations of all methods and of method RS showed low variabilities of results. The quotients S/σ_{pt} were below 1,0. The robust standard deviations are in the range of established values for the reproducibility standard deviation of the applied methods (see 3.4.2 value by precision experiments and 3.4.3 value by perception). The comparability of results is given. This conclusion is limited for the evaluation across the methods, because there were only a few results for some methods.*

The robust means of the evaluations were 143% (X_{ALL}) and 148% (X_{RS}) of the spiking level of gluten to sample B and thus within the recommendations for the applied methods (s. 3.4.3 and "recovery rates for gluten", p.57).

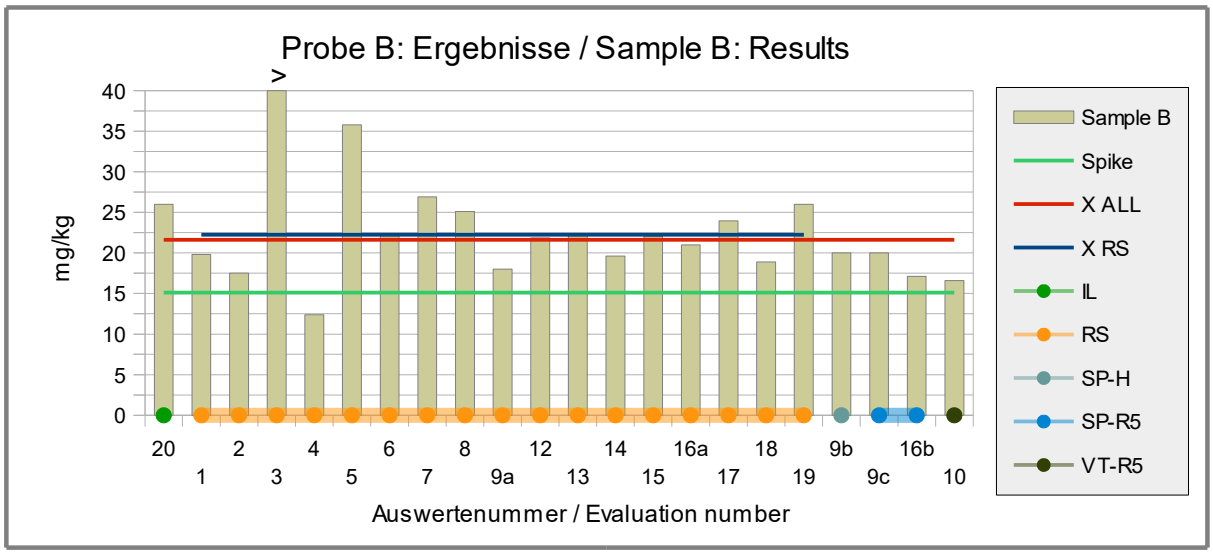


Abb./Fig. 23: ELISA Results Gluten
green line = Spiking level (Spike)
red line = Assigned value robust mean all results
blue line = Assigned value robust mean method RS
round symbols = Applied methods (see legend)

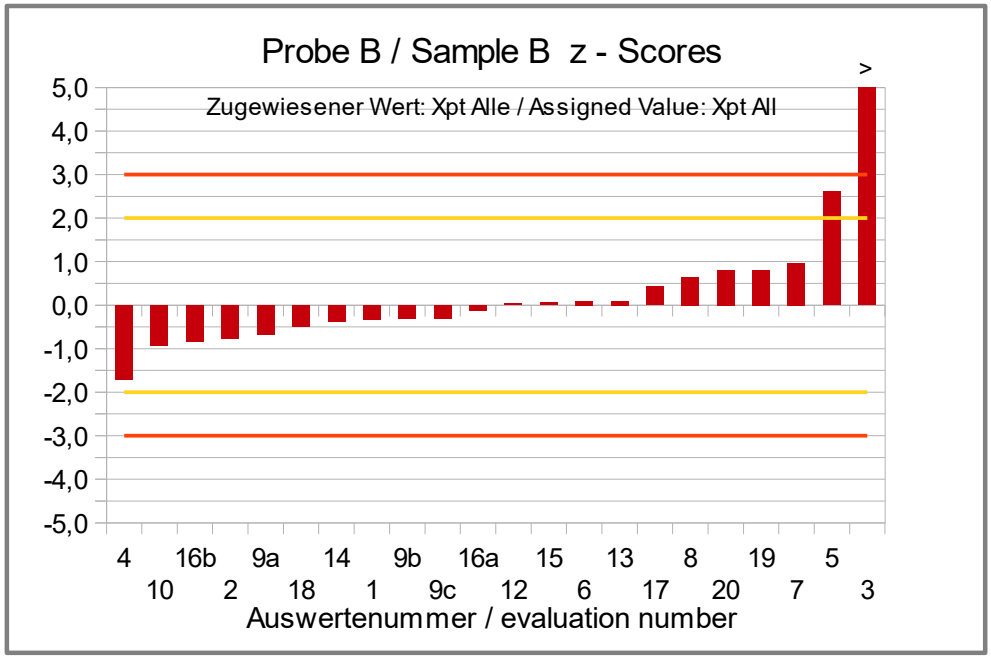


Abb./Fig. 24:
z-Scores (ELISA Results Gluten) Assigned value robust mean of all results

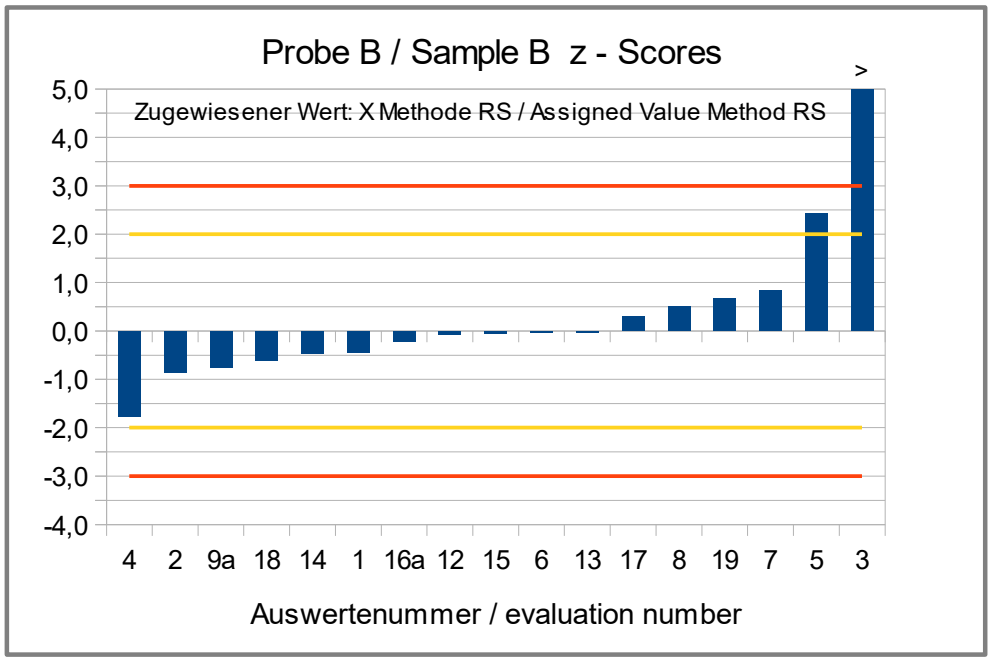
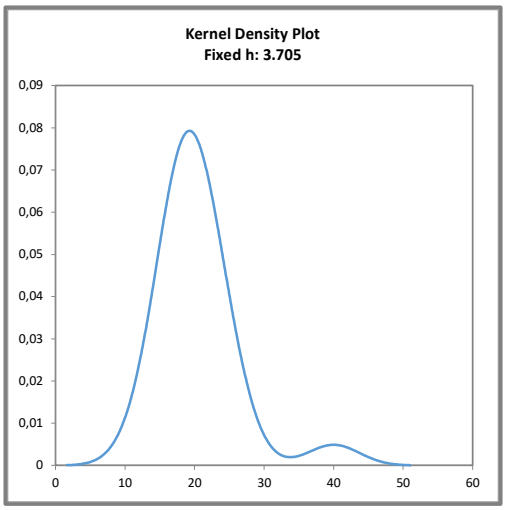


Abb./Fig. 25:
z-Scores (ELISA Results Gluten) Assigned value robust mean of results method RS (R-Biopharm, Ridascreen)

Quantitative valuation of ELISA results: Spiking level sample

Evaluation number	Gluten	z-Score Xpt_{ALL}	z-Score Xpt_{RS}	Method	Remarks
	[mg/kg]				
20	40,0	4,1		IL	
1	18,1	-0,34	-0,22	RS	
2	23,0	0,66	0,80	RS	
3	15,7	-0,82	-0,72	RS	
4	12,7	-1,4	-1,4	RS	
5	17,8	-0,39	-0,28	RS	
6	16,4	-0,68	-0,58	RS	
7	20,8	0,21	0,34	RS	
8	24,7	0,99	1,1	RS	
9a	18,0	-0,36	-0,24	RS	
12	16,1	-0,74	-0,64	RS	
13	18,8	-0,20	-0,08	RS	
14	20,1	0,07	0,19	RS	
15	19,0	-0,15	-0,03	RS	
16a	23,2	0,70	0,84	RS	
17	20,9	0,23	0,36	RS	
18	17,6	-0,44	-0,33	RS	
19	22,0	0,45	0,59	RS	
9b	25,0	1,1		SP-H	
9c	21,0	0,25		SP-R5	
16b	18,0	-0,36		SP-R5	
10	19,0	-0,15		VT-R5	



Methods:

IL = Immunolab
RS = Ridascreen®, R-Biopharm
SP-H = SensiSpec INgezim Hydrolysed Gluten, Eurofins
SP-R5 = SensiSpec INgezim Gluten R5, Eurofins
VT-R5 = Veratox, Neogen

Abb. / Fig. 26:
Kerndichte-Schätzung aller ELISA-Ergebnisse (mit h = 0,75 x σpt von Xpt_{ALL})

Kernel density plot of all ELISA results (with h = 0,75 x σpt of Xpt_{ALL})

Comments:
The kernel density estimation shows nearly a symmetrical distribution of results with a small side peak at 40 mg/kg, due to a single value outside the target range (method IL).

Characteristics: Quantitative evaluation ELISA Gluten

Spiking level sample

Statistic Data	**All Results** *[mg/kg]*	**Method RS** *[mg/kg]*
Assigned value (X_{pt})	$X_{pt_{ALL}}$	$X_{pt_{METHOD\ RS}}$
Number of results	22	17
Number of outliers	-	0
Mean	20,4	19,1
Median	19,0	18,8
Robust Mean (X_{pt})	**19,8**	**19,2**
Robust standard deviation (S*)	**3,48**	**3,20**
Target range:		
Target standard deviation σ_{pt}	**4,94**	**4,79**
lower limit of target range	**9,88**	**9,58**
upper limit of target range	**29,6**	**28,7**
Quotient S/σ_{pt}*	*0,70*	*0,67*
Standard uncertainty $U_{(Xpt)}$	*0,927*	*0,971*
Results in the target range	*21*	*17*
Percent in the target range	*95*	*100*

Methods:
RS = R-Biopharm, Ridascreen®

Comments to the statistical characteristics and assigned values:

The kernel density estimation showed almost a symmetrical distribution of results without clear method-dependent differences.

The evaluation of results of all methods as well as the results of method RS showed a low variability of results, respectively. The quotients S/σ_{pt} were below 1,0. The robust standard deviations are in the range of established values for the repeatability and reproducibility standard deviation of the applied methods (see 3.4.2 value by precision experiments and 3.4.3 value by perception). The comparability of results is given.*
This conclusion is limited for the evaluation across the methods, because there were only a few results for some methods.

The robust means of the evaluations were 135% (X_{ALL}) and 131% (X_{RS}) of the spiking level of gluten to the spiking level sample and thus within the recommendations for the applied methods (s. 3.4.3 and "recovery rates for gluten", p.57).

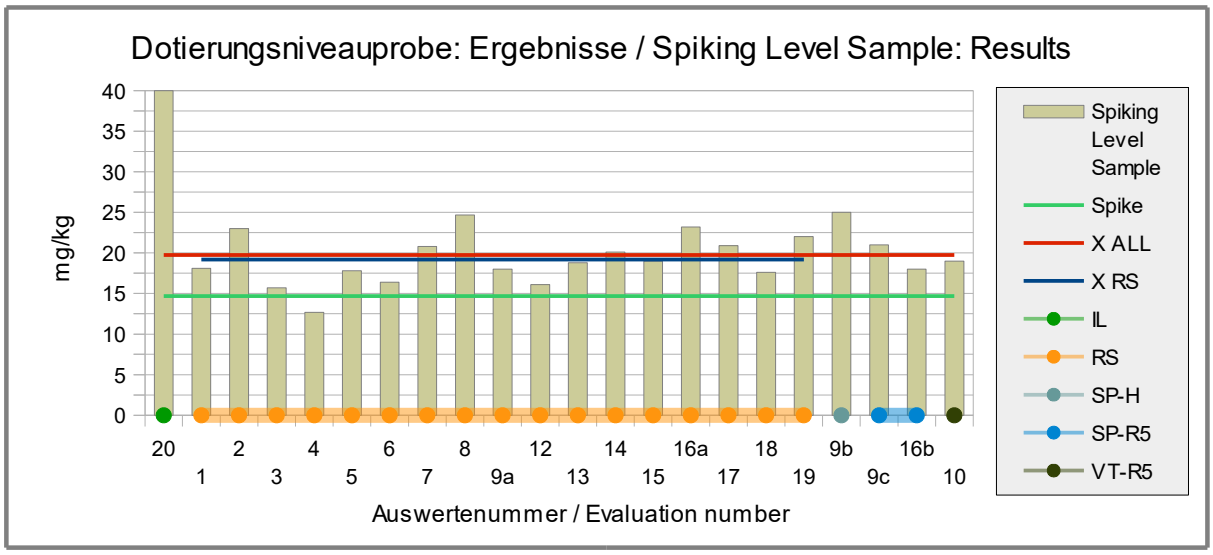


Abb./Fig. 27: ELISA Results Gluten
green line = Spiking level (Spike)
red line = Assigned value robust mean all results
blue line = Assigned value robust mean method RS
round symbols = Applied methods (see legend)

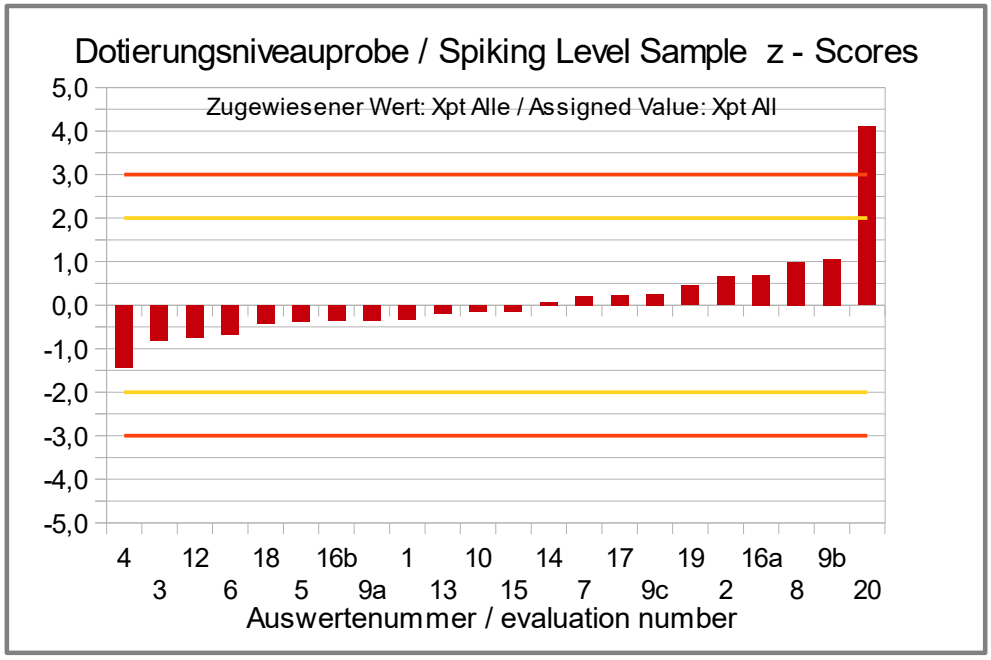


Abb./Fig. 28:
z-Scores (ELISA Results Gluten) Assigned value robust mean of all results

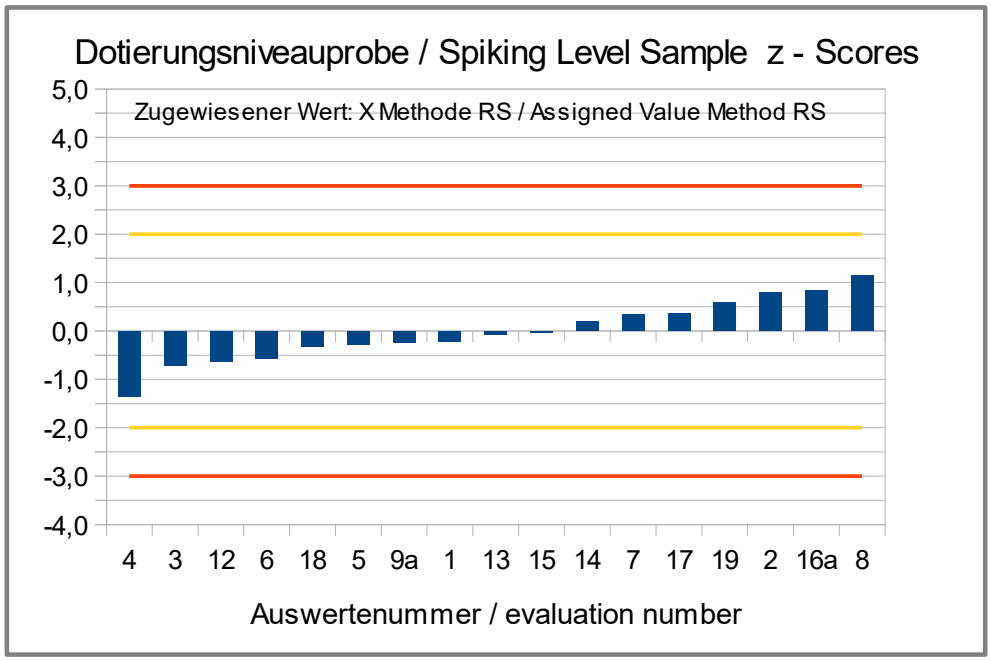


Abb./Fig. 29:
z-Scores (ELISA Results Gluten) Assigned value robust mean of results method RS (R-Biopharm, Ridascreen)

Recovery Rates with z-Scores for Gluten: Spiking level sample and Sample B

Evaluation number	Spiking Level Sample	Recovery rate*		Sample B	Recovery rate*		Method	Remarks
	[mg/kg]	[%]	[Z_{RR}]	[mg/kg]	[%]	[Z_{RR}]		
20	40,0	272	6,9	26,0	172	2,9	IL	
1	18,1	**123**	0,93	19,8	**131**	1,2	RS	
2	23,0	156	2,3	17,5	**116**	0,64	RS	
3	15,7	**107**	0,27	164	1083	39	RS	
4	12,7	**86**	-0,55	12,4	**82**	-0,72	RS	
5	17,8	**121**	0,85	35,8	237	5,5	RS	
6	16,4	**112**	0,46	22,1	**146**	1,9	RS	
7	20,8	**141**	1,7	26,9	178	3,1	RS	
8	24,7	168	2,7	25,1	166	2,6	RS	
9a	18,0	**122**	0,90	18,0	**119**	0,77	RS	
12	16,1	**110**	0,38	21,9	**145**	1,8	RS	
13	18,8	**128**	1,1	22,1	**146**	1,9	RS	
14	20,1	**137**	1,5	19,6	**130**	1,2	RS	
15	19,0	**129**	1,2	22,0	**146**	1,8	RS	
16a	23,2	158	2,3	21,0	**139**	1,6	RS	
17	20,9	**142**	1,7	24,0	159	2,3	RS	
18	17,6	**120**	0,79	18,9	**125**	1,0	RS	
19	22,0	**150**	2,0	26,0	172	2,9	RS	
9b	25,0	170	2,8	20,0	**132**	1,3	SP-H	
9c	21,0	**143**	1,7	20,0	**132**	1,3	SP-R5	
16b	18,0	**122**	0,90	17,1	**113**	0,53	SP-R5	
10	19,0	**129**	1,2	16,6	**110**	0,40	VT-R5	

RA**	50-150 %	RA**	50-150 %
Number in RA	**17**	Number in RA	**15**
Percent in RA	**77**	Percent in RA	**68**

* Recovery rate 100% relative size: Gluten, s. page 5

** Range of acceptance of AOAC for allergen ELISAS

Methods:

IL = Immunolab

RS = Ridascreen®, R-Biopharm

SP-H = SensiSpec INgezim Hydrolysed Gluten, Eurofins

SP-R5 = SensiSpec INgezim Gluten R5, Eurofins

VT-R5 = Veratox, Neogen

Comments:

For the spiking level sample 77% (17) of the participants obtained a recovery rate within the range of the AOAC-recommendation of 50-150%. For the spiked food matrix sample B 68% (15) of the obtained recovery rates were within the recommended range.

The related z-scores are based on the target standard deviation of 25%.

4.2.2 PCR Results: Gluten-containing Cereals (Wheat)

Qualitative valuation of results: Samples A and B

Evaluation number	Sample A	Sample A	Sample B	Sample B	Qualitative Valuation	Method	Remarks
	pos/neg	[mg/kg]	pos/neg	[mg/kg]	Agreement with consensus value		
10a	negative	-	positive	-	2/2 (100%)	FP	
10b	negative	-	positive	-	2/2 (100%)	GR	
10c	negative	-	positive	-	2/2 (100%)	SFA-ID	
9	negative		positive		2/2 (100%)	div	

	Sample A		Sample B	
Number positive	0		4	
Number negative	4		0	
Percent positive	0		100	
Percent negative	100		0	
Consensus value	negative		positive	
Spiking	negative		positive	

Methods:
FP = foodproof Detection Kit, BIOTECON Diagnostics
GR = SPECIALfinder Assay, Generon
SFA-ID = Sure Food Allergen ID, R-Biopharm / Congen
div = keine genaue Angabe / andere Methode
div = not indicated / other method

Comments:
The consensus values are in qualitative agreement with the spiking of sample B.

Qualitative Valuation PCR: Spiking Level Sample

Evaluation number	Gluten-cont. Cereals	Gluten-cont. Cereals	z-Score Xpt_{ALL}	Methods	Remarks
	pos/neg	[mg/kg]			
10a	positive			FP	
10b	positive			GR	
10c	positive			SFA-ID	
9	positive			div	

Number positive	4
Number negative	0
Percent positive	100
Percent negative	0
Consensus value	positive

Methods:
FP = foodproof Detection Kit, BIOTECON Diagnostics
GR = SPECIALfinder Assay, Generon
SFA-ID = Sure Food Allergen ID, R-Biopharm / Congen
div = keine genaue Angabe / andere Methode
div = not indicated / other method

Comments:
For the spiking level sample there were 100% positive results.

4.3 Participant z-Scores: overview table

Z-Scores for the assigned values from participants results (consensus values)

Evaluation number	ELISA β-Lacto-globulin: **Xpt** (div. Methods)		ELISA β-Lacto-globulin: **Xpt** (Method RS-F)		ELISA Casein: **Xpt** (div. Methods)		ELISA Casein: **Xpt** (Method RS-F)	
	Sample B*	Spiking Level Sample	Sample B	Spiking Level Sample	Sample B° #	Spiking Level Sample	Sample B #	Spiking Level Sample
1					-2,2	-0,01	-2,0	-0,25
2	-2,7	0,35	-2,8	-0,47	1,4	0,25	5,1	0,00
3					-1,9	2,9	-1,4	2,5
4	0,66	0,65			3,7	-0,26		
5								
6					-1,3		-0,28	
7		-0,28						
8		-1,5			1,7	-1,2		
9	-0,14	-0,17			2,1	-1,7		
10		-2,9			-1,6	1,1		
11					-1,2	-2,3		
12		-1,2						
13	0,28	0,55	0,08	-0,32	-1,0	0,11	0,28	-0,13
14	1,0	0,93	0,78	-0,01	3,8	0,25	9,7	0,00
15	0,92	1,8	0,68	0,66	-2,2	-0,10	-1,9	-0,33
16/16a	-1,3	0,33			0,37	-0,12		
16b					-0,50	0,66		
17					-0,09	-0,09		
18	0,25	1,1	0,05	0,15	0,16	-0,70		
19		-0,71			0,33	4,7	2,9	4,2
20								

Methods: * PEAK 11 = INgezim (Ingenasa), ELISA Kit II (Morinaga Inst.), Ridascreen® Fast (R-Biopharm)
RS-F = Ridascreen® Fast (R-Biopharm)
informativ
° z'-Score

Bewertung des z-Scores / valuation of z-score (DIN ISO 13528:2009-01):
-2 ≤ z-score ≤ 2 erfolgreich / successful (in green)
-2 > z-score > 2 „Warnsignal“ / warning signal (in yellow)
-3 > z-score > 3 „Eingriffssignal“ / action signal (in red)

Z-Scores for the assigned values from participants results (consensus values)

Evaluation number	ELISA Milk: **Xpt** (div. Methods)		ELISA Gluten: **Xpt** (div. Methods)		ELISA Gluten: **Xpt** (Method RS)	
	Sample B	Spiking Level Sample	Sample B	Spiking Level Sample	Sample B	Spiking Level Sample
1			-0,34	-0,34	-0,44	-0,22
2			-0,76	0,66	-0,85	0,80
3			26	-0,82	25	-0,72
4			-1,7	-1,4	-1,8	-1,4
5			2,6	-0,39	2,4	-0,28
6			0,09	-0,68	-0,03	-0,58
7			0,97	0,21	0,84	0,34
8			0,64	0,99	0,51	1,1
9a			-0,67	-0,36	-0,76	-0,24
9b			-0,30	1,1		
9c			-0,30	0,25		
10			-0,93	-0,15		
11						
12			0,04	-0,74	-0,07	-0,64
13			0,09	-0,20	-0,03	-0,08
14			-0,38	0,07	-0,48	0,19
15			0,07	-0,15	-0,04	-0,03
16/16a			-0,12	0,70	-0,22	0,84
16b			-0,84	-0,36		
17			0,43	0,23	0,31	0,36
18			-0,50	-0,44	-0,60	-0,33
19			0,81	0,45	0,67	0,59
20			0,81	4,1		

Methods: RS = Ridascreen®, R-Biopharm

Bewertung des z-Scores / valuation of z-score (DIN ISO 13528:2009-01):
-2 ≤ z-score ≤ 2 erfolgreich / successful (in green)
-2 > z-score > 2 „Warnsignal“ / warning signal (in yellow)
-3 > z-score > 3 „Eingriffssignal“ / action signal (in red)

z-Scores for the assigned values from spiking level (recovery rates)

Evaluation number	ELISA β-Lacto-globulin: **Xpt** (spiked level)		ELISA Casein: **Xpt** (spiked level)		ELISA Milch: **Xpt** (spiked level)		ELISA Gluten: **Xpt** (spiked level)	
	Sample B	Spiking Level Sample	Sample B	Spiking Level Sample	Sample B	Spiking Level Sample	Sample B	Spiking Level Sample
1			-3,4	1,8			1,2	0,93
2	-3,5	-1,9	-1,1	2,2	10	6,6	0,64	2,3
3			-3,2	6,1	-3,4	13	39	0,27
4	-2,3	-1,8	0,30	1,5			-0,72	-0,55
5					-3,3	-0,59	5,5	0,85
6			-2,8				1,9	0,46
7	0,64	-2,2					3,1	1,7
8	-3,3	-2,8	-0,90	0,10			2,6	2,7
9/9a	-2,2	-2,2	-0,65	-0,62	-3,3	-2,0	0,77	0,90
9b							1,3	2,8
9c							1,3	1,7
10	-3,9	-3,5	-3,0	3,5	-3,7	-1,5	0,40	1,2
11	-3,9		-2,7	-1,6				
12	0,72	-2,7					1,8	0,38
13	-1,9	-1,8	-2,6	2,0	-2,3	-1,7	1,9	1,1
14	-1,5	-1,6	0,39	2,2			1,2	1,5
15	-1,50	-1,2	-3,3	1,7			1,8	1,2
16/16a	-2,8	-1,9	-1,8	1,7	11	14	1,6	2,3
16b			-2,3	2,8			0,53	0,90
17			-2,0	1,7			2,3	1,7
18	-2,0	-1,5	-1,9	0,83			1,0	0,79
19	1,1	-2,4	-1,8	8,7			2,9	2,0
20					-3,0	-1,3	2,9	6,9

Bewertung des z-Scores / valuation of z-score (DIN ISO 13528:2009-01):
-2 ≤ z-score ≤ 2 erfolgreich / successful (in green)
-2 > z-score > 2 „Warnsignal“ / warning signal (in yellow)
-3 > z-score > 3 „Eingriffssignal“ / action signal (in red)

5. Documentation

5.1 Details by the participants

Note: Information given in German were translated by DLA to the best of our knowledge (without guarantee of correctness).

5.1.1 ELISA: β-Lactoglobulin

Meth. Abbr.	Evaluation no.	Date of Analysis	Result Sample A		Result Sample B		Result Spiking Level Sample		NWG / LOD *	BG / LOQ *	MU*	quantitative Result Given as	Method
		day/month	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	mg/kg	mg/kg	%	e.g. food/ protein	ELISA Test-Kit + Manufacturer
AQ	8	12.05.21	positive	1,41	positive	5,52	positive	9,22	9,1	0,4	30	beta-Lactoglobulin	AgraQuant ELISA β-Lactoglobulin COLAL1048, RomerLabs
AQ	10	07/June	negative	<0,01	positive	0,34	positive	3,95	0,0015	0,01	15	beta-Lactoglobulin	AgraQuant ELISA β-Lactoglobulin COLAL1048, RomerLabs
AQ	11		-		positive	0,35	positive	> 0,4	0,01	0,01		beta-Lactoglobulin	AgraQuant ELISA β-Lactoglobulin COLAL1048, RomerLabs
IN	16	21.06.21	positive	0,8	positive	>4,0 (7,7*)	positive	>4,0 (15,8*)		0,1		beta-Lactoglobulin	ingezim b-lactoglobulina 30.blg.k.2
MI-II	4	06.05.21	positive	37,51	positive	132,59	positive	169,78	NA	0,31	NA	Milk proteins, total	Morinaga Beta-lactoglobulin ELISA Kit II (M2112)
MI-II	9	19.05.21	positive	0,86	positive	11	positive	14	0,031	0,031		beta-Lactoglobulin	Morinaga Beta-lactoglobulin ELISA Kit II (M2112)
RS-C	7		-	7,3	-	33,5	-	13,6	1,4	5		beta-Lactoglobulin	Ridascreen® β-Lactoglobulin R4901, R-Biopharm
RS-C	12	10.05.21	negative	<5	positive	26,65	positive	10,12	2,1	5		beta-Lactoglobulin	Ridascreen® β-Lactoglobulin R4901, R-Biopharm
RS-C	19		-		positive	29	positive	12		5		beta-Lactoglobulin	Ridascreen® β-Lactoglobulin R4901, R-Biopharm
RS-F	2	20.05.21	positive	0,57	positive	3,64	positive	15,9	0,04	0,167	20	beta-Lactoglobulin	Ridascreen® FAST β-Lactoglobulin R4902, R-Biopharm
RS-F	13	16.06.21	positive	0,345	positive	12,2	positive	16,6		0,167		beta-Lactoglobulin	Ridascreen® FAST β-Lactoglobulin R4902, R-Biopharm
RS-F	14		negative	< 0,167	positive	14,3	positive	18	0,04	0,167		beta-Lactoglobulin	Ridascreen® FAST β-Lactoglobulin R4912, R-Biopharm
RS-F	15	21-June	negative	< 0,5	positive	14	positive	21		0,5		beta-Lactoglobulin	Ridascreen FAST B-lactoglobulin R4912, R-Biopharm
RS-F	18	21.05.21	positive	0,58	positive	12,1	positive	18,7	0,17	0,17		beta-Lactoglobulin	Ridascreen® FAST β-Lactoglobulin R4902, R-Biopharm

* NWG Nachw eisgrenze / BG Bestimmungsgrenze

Continuation ELISA β-Lactoglobulin:

Meth. Abbr.	Evaluation no.	Specifity	Remarks to the Method (Extraction and Determination)	Method Accredited ISO/IEC 17025	Further Remarks
		Antibody	e.g. Extraction Solution / Time / Temperature	yes/no	
AQ	8			yes	
AQ	10			yes	
AQ	11			no	
IN	16			YES	
MI-II	4		as stipulated in kit insert, overnight extraction, room temperature	yes	low recovery in sample A (29%) dilution 1/20 reported unable to quantify recovery in Sample B
MI-II	9	detects cow 's milk ßLac	as per kit instructions	yes	*10 = total milk protein
RS-C	7			no	
RS-C	12		as per kit instructions	no	
RS-C	19		Test can underestimate native ß-Lactoglobulin.	yes	
RS-F	2	cow 's milk (cross reaction: sheep, goat, buffalo)	Extraktor2, 10 min bei 100°C; Alleregenextraktionspuffer; 10 min bei 60°C	yes	
RS-F	13			No	sample futher dilluted 1:10
RS-F	14				ELISA-Test: R4912
RS-F	15			yes	
RS-F	18	beta-Lactoglobulin		yes	

5.1.2 ELISA: Casein

Meth. Abbr.	Evaluation no.	Date of Analysis	Result Sample A		Result Sample B		Result Spiking Level Sample		NWG / LOD *	BG / LOQ *	MU*	quantitative Result Given as	Method
		day/month	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	mg/kg	mg/kg	%	e.g. food/ protein	ELISA Test-Kit + Manufacturer
AQ	11		negative	<0,2	positive	4,9	positive	9,4	0,2	1		Casein	AgraQuant Casein COKAL 1200, RomerLabs
AQ	17	24.06.21	negative	<1	positive	7,6	positive	22,07	0,04	1		Casein	AgraQuant Casein COKAL 1200, RomerLabs
AQ	18	24.06.21	positive	0,46	positive	8,2	positive	18,6	0,2	0,2		Casein	AgraQuant Casein COKAL 1200, RomerLabs
IL	10	27/May	negative	<0,2	positive	3,84	positive	29	0,04	0,2	15	Casein	Immunolab Casein ELISA
IN	16a	18.06.21	negative	<0,3	positive	>6,0 (8,7*)	positive	>6,0 (21,9*)		0,3		Casein	ingezim caseina 30.blg.k.2
MI-I	8	11.05.21	negative		positive	15,1	positive	19,8	0,625		30	Milk proteins, total	Morinaga Casein ELISA Kit (M2102)
MI-II	4	06.05.21	negative	<0,78	positive	20,85	positive	26,35	NA	0,31	NA	Milk proteins, total	Morinaga Casein ELISA Kit II (M2113)
MI-II	9	17.05.21	negative	<0,25	positive	13	positive	13	0,25	0,25		Casein	Morinaga Casein ELISA Kit II (M2113)
RS-F	1	04.05.2021	-	<0.5	-	2,5	-	22,5				Casein	Ridascreen® FAST Casein R4612, R-Biopharm
RS-F	2	12.05.21	positive	2,7	positive	11,3	positive	24	0,12	0,5	25	Casein	Ridascreen® FAST Casein R4612, R-Biopharm
RS-F	3	25.05.21	negative		positive	3,2	positive	38,7	2,5			Casein	Ridascreen® FAST Casein R4612, R-Biopharm
RS-F	6		negative	<0.5	positive	4,6	positive	>13.5	0,24	0,5		Casein	Ridascreen® FAST Casein R4612, R-Biopharm
RS-F	13		negative	<0,5		5,3	-	23,2		2,5		Casein	Ridascreen® FAST Casein R4612, R-Biopharm
RS-F	14		negative	< 2,5	positive	17	positive	24	0,12	2,5		Casein	Ridascreen® FAST Casein R4612, R-Biopharm
RS-F	15	21-June	negative	< 0,5	positive	2,6	positive	22		0,5		Casein	Ridascreen® FAST Casein R4612, R-Biopharm
RS-F	19		-		positive	8,6	positive	49		2,5		Casein	Ridascreen® FAST Casein R4612, R-Biopharm
SP	16b	18.06.21	negative	<0,2	positive	6,6	positive	26,3		0,2		Casein	SensiSpec ELISA Casein, Eurofins

* NWG Nachweisgrenze / BG Bestimmungsgrenze

* LOD limit of detection / LOQ limit of quantitation

* MU Messunsicherheit / MU measurement uncertainty

Meth. Abbr.	Evaluation no.	Specifity	Remarks to the Method (Extraction and Determination)	Method Accredited ISO/IEC 17025	Further Remarks
		Antibody	e.g. Extraction Solution / Time / Temperature	yes/no	
AQ	11			no	
AQ	17	Casein	Extraction solution 15 min at 60°C, 1:10 dilution	no	
AQ	18	Casein		yes	
IL	10			yes	
IN	16a			YES	
MI-I	8				
MI-II	4		as stipulated in kit insert, overnight extraction, room temperature	yes	recovery in sample A (78%) recovery in sample B (73%)
MI-II	9	detects cow's milk casein	as per kit instructions	yes	*1,24 = total milk protein
RS-F	1				
RS-F	2	cow's milk (cross-reaction: sheep, goat, buffalo)	Extractor2, 10 min at 100°C; Allergen extraction buffer; 10 min at 60°C	yes	
RS-F	3			yes	
RS-F	6				
RS-F	13			No	sample not futher dilluted
RS-F	14				
RS-F	15			yes	
RS-F	19			yes	
SP	16b			NO	

5.1.3 ELISA: Milk

Meth. Abbr.	Evaluation no.	Date of Analysis	Result Sample A		Result Sample B		Result Spiking Level Sample		NWG / LOD *	BG / LOQ *	MU*	quantitative result given as	Method
		day/month	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	mg/kg	mg/kg	%	e.g. food/ protein	ELISA Test-Kit + Manufacturer
RS-F	2	19.05.21	positive	8,2	positive	218,2	positive	202,5	0,7	2,5	20	Milk proteins, total	Ridascreen® FAST Milk R4652, R-Biopharm
RS-F	3	21.05.21	positive	7,1	positive	16,3	positive	316,8	3			Milk proteins, total	Ridascreen® FAST Milk R4652, R-Biopharm
RS-F	6		positive	6,5	positive	>67.5	positive	>67.5	1,5	2,5		Milk proteins, total	Ridascreen® FAST Milk R4652, R-Biopharm
RS-F	13	21.06.21	positive	9,8	positive	36,3	-	43,16		2,5		Milk proteins, total	Ridascreen® FAST Milk R4652, R-Biopharm
RS-F	16	24.06.21	-	-	positive	>67,5 (225,6*)	positive	>67,5 (344,8*)	0,7	2,5		Milk proteins, total	Ridascreen® FAST Milk R4652, R-Biopharm
SP	9	04.06.21	positive	0,91	positive	12	positive	39	0,4	0,4		Milchproteine, gesamt	other: please fill in!
SP	20	17.06.21	positive	0,8	positive	15,6	positive	51.8	0,05	0,4		Casein + ß-Lactoglobulin	SENSISpec Milk ELISA
VT	5	03.06.21	-	< LOQ	-	10,96	-	65	1	2	22	Milk proteins, total	Veratox Total Milk Allergen, Neogen
VT	10	18/May	negative	<2,5	positive	12,5	positive	135	1	2,5	18	Skimmed milk powder	Veratox Total Milk Allergen, Neogen

* NWG Nachw eisgrenze / BG Bestimmungsgrenze
* LOD limit of detection / LOQ limit of quantitation
* MU Messunsicherheit / MU measurement uncertainty

Meth. Abbr.	Evaluation no.	Specifity	Remarks to the Method (Extraction and Determination)	Method Accredited ISO/IEC 17025	Further Remarks
		Antibody	e.g. Extraction Solution / Time / Temperature	yes/no	
RS-F	2	cow 's milk (cross reaction: sheep, goat, buffalo)	Extractor2, 10 min at 100°C; Allergen extraction buffer; 10 min at 60°C	yes	
RS-F	3			yes	
RS-F	6				
RS-F	13			No	sample futher dilluted 1:2
RS-F	16			YES	Sample A: non-reproducibles results
SP	9		as per kit instructions	yes	SENSISpec Milch HU0030038
SP	20	Casein + BLG			
VT	5	-	estrazione : PBS 10mM + extraction additive 15 m a 60 °C / incubazione 30 m / saggio eseguito a t amb	yes	Alni
VT	10			yes	

5.1.4 ELISA: Gluten

Meth. Abbr.	Evaluation no.	Date of Analysis	Result Sample A		Result Sample B		Result Spiking Level Sample		NWG / LOD *	BG / LOQ *	MU*	quantitative Result given as	Method
		day/month	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	mg/kg	mg/kg	%	e.g. food/ protein	ELISA Test-Kit + Manufacturer
IL	20	04.05.21	negative	0	positive	26	positive	40	0,6	4		Gluten	Immunolab Gliadin ELISA
RS	1	10.05.21	-	<3.0	-	19,8	-	18,1				Gluten	Ridascreen
RS	2	20.05.21	negative	<5	positive	17,5	positive	23	1	5	20	Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	3	06.05.21	negative		positive	163,5	positive	15,7	5			Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	4	27.05.21	negative	<5	positive	12,39	positive	12,67	NA	5	NA	Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	5	18.05.21	-	< LOQ	-	35,77	-	17,82	1	5	10	Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	6		negative	<10	positive	22,1	positive	16,4	3	10		Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	7		-	<5	-	26,9	-	20,8	1	5		Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	8	15.05.21	negative		positive	25,09	positive	24,67		5	30	Gluten	
RS	9a	04.06.21	negative	<5	positive	18	positive	18	3	5		Gluten	andere: bitte eingeben!
RS	12	17.05.21	negative	<5	positive	21,86	positive	16,11	1	5		Gluten	Ridascreen Gliadin R 7001, R-Biopharm
RS	13	24.06.21	negative	<6,6	positive	22,11	-	18,78		6,6		Gluten	RIDASCREEN R7001
RS	14		negative	< 5,0	positive	19,6	positive	20,1	1	5		Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	15	22-June	negative	< 5	positive	22	positive	19		5		Gluten	Ridascreen Gliadin R7001, R-Biopharm
RS	16a	12.05.21	negative	<5,0	positive	21	positive	23,2		5		Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	17	24.06.21	negative	<5	positive	23,96	positive	20,88	1	5		Gluten	Ridascreen Gliadin R7001, R-Biopharm
RS	18	01.06.21	negative		positive	18,9	positive	17,6	5	5		Gluten	Ridascreen® Gliadin R7001, R-Biopharm
RS	19		-		positive	26	positive	22		5		Gluten	Ridascreen® Gliadin R7001, R-Biopharm
SP-H	9b	18.05.21	negative	<5	positive	20	positive	25	5	5		Gluten	andere: bitte eingeben!
SP-R5	9c	11.05.21	negative	<3,12	positive	20	positive	21	3,12	3,12		Gluten	andere: bitte eingeben!
SP-R5	16b	26/05/2002 1	negative	<5,0	positive	17,1	positive	18		3,12		Gluten	Sensispe Ingezim Gluten R5 30.glu.k.2
VT-R5	10	27/May	negative	<3,0	positive	16,6	positive	19	3	5	15	Gluten	Veratox Gliadin R5, Neogen

* NWG Nachw eisgrenze / BG Bestimmungsgrenze

* LOD limit of detection / LOQ limit of quantitation

* MU Messunsicherheit / MU measurement uncertainty

Continuation ELISA Gluten:

Meth. Abbr.	Evaluation no.	Specifity	Remarks to the Method (Extraction and Determination)	Method Accredited ISO/IEC 17025	Further Remarks
		Antibody	e.g. Extraction Solution / Time / Temperature	yes/no	
IL	20				
RS	1				
RS	2	Gliadin from w heat (and prolamins from rye, barley, spelt)	Coctail solution, 40min at 50°C; then EtOH(60%) 1 hr at RT	yes	
RS	3			yes	
RS	4		as stipulated in kit insert, ext 2021-05-26, ELISA 2021-05-27	yes	recovery in sample A (71%) recovery in sample B (77%)
RS	5	anticorpo monoclonale R5	estrazione : cocktail solution (ridascreen R7006) a 50 °C x 45 m + etanolo 80 % / incu bazione 1h e 30 m / saggio eseguito a t amb	yes	AIni
RS	6				
RS	7		Extraction w ith Cocktail solution	no	
RS	8	R5	r-biopharm Ridascreen	yes	
RS	9a	R5 antibody of Mendez detects prolamins (gliadins) from w heat, rye and barley	as per kit instructions	yes	r-biopharm R7001
RS	12		as per kit instructions	yes	
RS	13			No	not tested
RS	14	monoclonal Ab 5			
RS	15	R5		yes	
RS	16a	R5		YES	
RS	17	Gliadin	Extraction w ith Cocktail solution and Ethanol, 40 min at 50°C, 1:500 dilution	no	
RS	18	Gliadins (R5-Antibody)		yes	
RS	19			yes	
SP-H	9b	monoclonal Antibody R5	as per kit instructions	yes	SENSISpec INgezim Gluten Hidrolizado 30.GLH.K2
SP-R5	9c	R5 antibody of Mendez detects prolamins (gliadins) from w heat, rye and barley	as per kit instructions	yes	SENSISpec INgezim Gluten 30.GLU.K2
SP-R5	16b	R5		YES	
VT-R5	10	R5		yes	

5.1.5 PCR: Gluten-containing Cereals

Meth. Abbr.	Evaluation no.	Date of Analysis	Result Sample A		Result Sample B		Result Spiking Level Sample		NWG / LOD *	BG / LOQ *	MU*	quantitative Result given as	Method
		day/month	qualitative	mg/kg	qualitative	mg/kg	qualitative	mg/kg	mg/kg	mg/kg	%	e.g. food/ protein	PCR Test-Kit + Manufacturer
FP	10a	14/June	negative	-	positive	-	positive	-				Gluten	foodproof Detection Kit, BIOTECON Diagnostics
GR	10b	25/May	negative	-	positive	-	positive	-				Gluten	SPECIALfinder Assay, real time PCR, Generon
SFA-ID	10c	16/June	negative	-	positive	-	positive	-				Gluten	Sure Food Allergen ID, R-Biopharm / Congen
div	9	03.06.21	negative		positive		positive		5			Wheat DNA	Selection PCR-Methods

* NWG Nachweisgrenze / BG Bestimmungsgrenze
* LOD limit of detection / LOQ limit of quantitation
* MU Messunsicherheit / MU measurement uncertainty

Meth. Abbr.	Evaluation no.	Specifity	Remarks to the Method (Extraction and Determination)	Method Accredited ISO/IEC 17025	Further Remarks
		Target-Sequence / -DNA	e.g. Extraction / enzymes / clean-up / real time PCR / gel electrophoresis / cycles	yes/no	
FP	10a				
GR	10b			yes	
SFA-ID	10c				
div	9		CTAB / Proteinase K / Amylase A / Promega Maxwell / Real-time PCR / 45 Cycles	yes	

5.2 Homogeneity

5.2.1 Mixture homogeneity before bottling

Microtracer Homogeneity Test
DLA-ptAL03 Sample B

Gewicht Gesamtprobe	2,51	kg
Microtracer	FSS-rot lake	
Teilchengröße	75 – 300	µm
Gewicht pro Partikel	2,0	µg
Tracerzugabe	21,7	mg/kg

Analysenergebnisse:

Probe	Einwaage [g]	Partikel Anzahl	Partikel [mg/kg]
1	5,01	46	18,4
2	4,98	50	20,1
3	5,04	58	23,0
4	5,05	53	21,0
5	4,96	47	19,0
6	5,00	55	22,0
7	4,96	60	24,2
8	5,03	57	22,7

Poisson-Verteilung		
Probenanzahl	8	
Freiheitsgrad	7	
Mittelwert	53,2	Particles
Standardabweichung	5,13	Particles
χ^2 (CHI-Quadrat)	3,45	
Wahrscheinlichkeit	**84**	%
Wiederfindungsrate	98	%

Normal distribution		
Number of samples	8	
Mean	21,3	mg/kg
Standard deviation	2,05	mg/kg
rel. Standard deviaton	9,63	%
Horwitz standard deviation	10,1	%
HorRat-value	**1,0**	
Recovery rate	98	%

Microtracer Homogeneity Test
DLA-ptAL03 Spiking Level Sample

Weight whole sample	1,50	kg
Microtracer	FSS-rot lake	
Particle size	75 – 300	µm
Weight per particle	2,0	µg
Addition of tracer	27,5	mg/kg

Result of analysis

Sample	Weight [g]	Particle number	Particles [mg/kg]
1	4,99	59	23,6
2	5,05	45	17,8
3	5,00	60	24,0
4	5,05	53	21,0
5	4,99	44	17,6
6	4,97	57	22,9
7	5,02	64	25,5
8	5,05	55	21,8

Poisson distribution		
Number of samples	8	
Degree of freedom	7	
Mean	54,6	Particles
Standard deviation	7,16	Particles
χ^2 (CHI-Quadrat)	6,57	
Probability	**48**	%
Recovery rate	79	%

Normal distribution		
Number of samples	8	
Mean	21,8	mg/kg
Standard deviation	2,85	mg/kg
rel. Standard deviaton	13,1	%
Horwitz standard deviation	10,1	%
HorRat-value	**1,3**	
Recovery rate	79	%

5.3 Information on the Proficiency Test (PT)

Before the PT the participants received the following information in the sample cover letter:

PT number	***ptAL03 (2021)***
PT name	***Allergens III: β-Lactoglobulin, Casein and Gluten in hypoallergenic Infant Food with hydrolyzed Milk Protein***
Sample matrix (processing)	***Samples A + B:*** *Infant formula (powder) / Ingredients: maltodextrin, vegetable oils, soy protein isolate, hydrolyzed whey protein, hydrolyzed casein, vitamins, minerals and other additives and allergenic foods as skimmed milk powder and wheat flour (one of both samples)* ***Spiking Level Sample:*** *potato powder, other food additives and allergenic foods*
Number of samples and sample amount	*2 different Samples A + B: 25 g each* *+ 1 Spiking Level Sample: 15 g*
Storage	*Samples A, B + Spiking Level Sample:* *room temperature (PT period), cooled 2 - 10°C (long term)*
Intentional use	*Laboratory use only (quality control samples)*
Parameter	*qualitative + quantitative:* *β-Lactoglobulin, Casein and Gluten (Gluten-containing Cereals)* *Samples A + B: < 500 mg/kg* *Spiking Level Sample: < 500 mg/kg*
Methods of analysis	*Analytical methods are optional*
Notes to analysis	*The analysis of PT samples should be performed like a routine laboratory analysis.* *In general we recommend to homogenize a representative sample amount before analysis according to good laboratory practice, especially in case of low sample weights. Preferably, the total sample amount is homogenized.*
Result sheet	*One result each should be determined for Samples A and B and the Spiking Level Sample.* *The results should be filled in the result submission file.*
Units	*mg/kg*
Number of digits	*at least 2*
Result submission	*The result submission file should be sent by e-mail to:* ***pt@dla-lvu.de***
Last Deadline	***the latest June 25th 2021***
Evaluation report	*The evaluation report is expected to be completed 6 weeks after deadline of result submission and sent as PDF file by e-mail.*
Coordinator and contact person of PT	*Matthias Besler-Scharf PhD / Alexandra Scharf MSc.*

* Control of mixture homogeneity and qualitative testings are carried out by DLA. Any testing of the content, homogeneity and stability of PT parameters is subcontracted by DLA.

6. Index of participant laboratories in alphabetical order

Teilnehmer / Participant	Ort / Town	Land / Country
		AUSTRIA
		ITALY
		SWITZERLAND
		CANADA
		ITALY
		Germany
		Germany
		Germany
		FRANCE
		SPAIN
		Germany
		AUSTRIA
		Germany
		POLAND
		SPAIN
		Germany
		Germany
		Germany
		Germany
		Germany
		GREECE

[Die Adressdaten der Teilnehmer wurden für die allgemeine Veröffentlichung des Auswerte-Berichts nicht angegeben.]

[The address data of the participants were deleted for publication of the evaluation report.]

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